Role of neuronal nitric oxide synthase in human vascular tone and systemic haemodynamics in vivo

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ABSTRACT

Endothelial and neuronal nitric oxide synthase (eNOS and nNOS respectively) are constitutively expressed *in vivo*. Recent data showed that selective local inhibition of nNOS reduced basal blood flow without affecting endothelial-mediated vasodilatation induced by acetylcholine or increased shear stress - suggesting that eNOS and nNOS have distinct roles in vasoregulation. This thesis aimed to investigate the role of nNOS-derived nitric oxide (NO) in the regulation of skeletal blood flow during exercise and myocardial blood flow during increased cardiac workload. At a systemic level, the role of nNOS on blood pressure and haemodynamics was investigated in a first-in-man study. We used the non-selective NOS inhibitor, N^G-monomethyl-L-arginine (L-NMMA), and selective nNOS inhibitor, S-methyl-L-thiocitrulline (SMTC), to determine the role of eNOS and nNOS in opposing an increase in sympathetically mediated increases in arteriolar tone in the human forearm during handgrip exercise. We found that despite reducing basal forearm blood flow (FBF), intra-brachial L-NMMA or SMTC had no significant effect on the increase in FBF or conduit artery diameter induced by local handgrip exercise, even in the face of increased sympathetic stimulation with lower body negative pressure. We investigated the relative contribution of eNOS and nNOS in the regulation of coronary vascular tone during increasing metabolic demand as achieved through incremental cardiac pacing. We found that the pacing induced increase in coronary blood flow and artery diameter was blunted by intra-coronary L-NMMA but not so by SMTC. We then undertook the first investigation in humans of the effects of systemic nNOS inhibition on haemodynamics. We found that intravenous SMTC increased systemic vascular resistance and blood pressure, whilst stroke volume, cardiac output and heart rate were

reduced. Importantly, there was no effect on flow-mediated dilatation, an effect mediated by eNOS. These results suggest that nNOS has a major contribution to basal regulation of systemic vascular resistance in humans.

DECLARATION

I declare that I am the sole author of this report and that the work within is my own unless otherwise stated.

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+P<0.05 for HR at 15 min SMTC 3.0 μmol/Kg vs. SMTC 1.0 μmol/Kg; for DBP at 0min SMTC 3.0 μmol/Kg vs. SMTC 1.0 μmol/Kg; and for MAP at 0 min SMTC 3.0 μmol/Kg vs. SMTC 1.0 μmol/Kg.

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*P<0.05 for SV SMTC 3.0 μmol/Kg vs. placebo.

**P<0.01 CO and SVR SMTC 3.0 μmol/Kg vs. placebo.

+P<0.05 CO and SVR SMTC 3.0 μmol/Kg vs. SMTC 1.0 μmol/Kg.

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LIST OF ABBREVIATIONS

ACE	Angiotensin converting enzyme
ACh	Acetylcholine
ANOVA	Analysis of variance
APV	Average peak velocity
AT-1	Angiotensin II subtype 1 receptor
ATP	Adenosine triphosphate
BP	Blood pressure
BH_4	Tetrahydrobiopoterin
CBF	Coronary blood flow
cGMP	Cyclic guanosine-3',5-monophosphate
CNS	Central nervous system
CO	Cardiac output
CSA	Cross-sectional area
CVR	Coronary vascular resistance
D	Diameter
DBP	Diastolic blood pressure
DMD	Duchenne muscular dystrophy
DNA	Deoxyribonucleic acid
ECG	Electrocardiogram
EDHF	Endothelium-derived hyperpolarizing factors
EDRF	Endothelium-derived relaxing factor
EF	Ejection fraction
eNOS	Endothelial nitric oxide synthase

FBC	Full blood count
FBF	Forearm blood flow
FMD	Flow-mediated dilatation
GABA	Gamma-aminobutyric acid
GFR	Glomerular filtration rate
HR	Heart rate
iNOS	Inducible nitric oxide synthase
LAD	Left anterior descending artery
LBNP	Lower body negative pressure
LCx	Left circumflex artery
L-NA	N^G-nitro-L-arginine
L-NAA	N^G-amino-L-arginine
L-NAME	N^G-nitro-L-arginine methyl ester
L-NMMA	N^G-monomethyl-L-arginine
LV	Left ventricle/ left ventricular
L-VNIO	Vinyl-L-N^5-(1-imino-3-butenyl)-L-ornithine
LVOT	Left ventricular outflow tract
MAP	Mean arterial pressure
mdx	X-linked muscular dystrophy mutation
NADPH	Nicotinamide-adenine-dinucleotide phosphate
NANC	Non-adrenergic non-cholinergic
NE	Norepinephrine
NIRS	Near infra-red spectroscopy
NO	Nitric oxide
NOS	Nitric oxide synthase

nNOS	Neuronal nitric oxide synthase
N-PLA	N^W-propyl-L-arginine
NPY	Neuropeptide Y
N4S	N-(4S)-(4-amino-5-[aminoethyl] aminopentyl)-N-nitroguanidine
PDE-5	Phosphodiesterase-5
PET	Positron emission tomography
PGI_2	Prostacyclin
QCA	Quantitative coronary angiography
SBP	Systolic blood pressure
SMTC	S-methyl-L-thiocitrulline
SNP	Sodium nitroprusside
SV	Stroke volume
SVR	Systemic vascular resistance
SW	Stroke work
TXA_2	Thromboxane A_2
VIP	Vasoactive intestinal peptide
VTI	Velocity-time integral
2D	2 dimensional
3D	3 dimensional
7-NI	7-nitroindazole

CHAPTER 1:

INTRODUCTION

1.1 PHYSIOLOGICAL REGULATION OF VASCULAR TONE

1.1.1 Vascular tone

Appropriate vascular tone and its dynamic adjustment are important to ensure that perfusion mirrors the continuously changing metabolic requirements of tissues (Kharbanda and Deanfield, 2001; Li and Forstermann, 2000; Mombouli and Vanhoutte, 1999). At a given blood pressure, the blood flow to each organ is determined by the peripheral vascular resistance of the organ. This is adjusted by a variety of local mechanisms affecting the tone of the smooth muscle in the 'resistance' vessels – small terminal arteries and arterioles. The endothelium has a major influence on overall vascular tone by modulating the balance between endothelium-derived relaxing factors and contracting factors.

When the availability of oxygen to the tissues decreases (for example at altitude), the blood flow to the tissues increases. This vasodilatation also occurs in response to a decrease of other metabolites, such as glucose. In addition to this many humoral factors regulate vascular tone. These include vasoconstrictors, such as epinephrine and norepinephrine (NE) from the adrenal medullae and the sympathetic nervous system, and angiotensin, action of which is regulated by the kidneys.

1.1.2 The vascular endothelium

The endothelium is a dynamic organ with paracrine, endocrine and autocrine properties (Kharbanda and Deanfield, 2001; Vallance and Chan, 2001; Verma and Anderson, 2002). It plays a pivotal role in vascular homeostasis, which is achieved through a complex set of interactions between a host of endothelium-derived factors. These factors influence a broad range of mechanisms by acting on endothelial cells, as well as cells within the vascular lumen (primarily leucocytes and platelets) and in the sub-endothelial tissues (primarily vascular smooth muscle cells).

Endothelium-derived factors have three main broad types of action 1: maintaining vascular tone, through vasodilatation and vasoconstriction, 2: controlling vascular inflammation, by modulation of cell-to-cell interactions between leucocytes, platelets, smooth muscle cells and endothelial cells, and 3: regulating the coagulation/fibrinolysis axis, through the formation and dissolution of vascular thrombotic material (Kharbanda and Deanfield, 2001; Vallance and Chan, 2001; Verma and Anderson, 2002). Alterations in the normal function of the endothelium are associated with vascular as well as systemic disorders.

Relaxing factors include nitric oxide (NO), prostacyclin (PGI2), bradykinin and endothelium-derived hyperpolarizing factors (EDHF); and contracting factors include the endothelin group of peptides, angiotensin-II and thromboxane A_2 (TXA_2) (Davignon and Ganz, 2004; Duffy et al., 1999; Feletou and Vanhoutte, 1999; Miyauchi and Masaki, 1999; Taddei et al., 1999). Endothelial detection of local changes in

metabolism, pressure and flow determines the relative balance between production of endothelial relaxing and contracting factors (Kharbanda and Deanfield, 2001).

1.1.3 Autonomic nervous system

The autonomic nervous system is divided into sympathetic and parasympathetic systems, the main neurotransmitters being norepinephrine (NE) and acetylcholine (ACh). The vasomotor centre is located in the reticular substance of the medulla and the lower third of the pons. A large number of areas in the brain can excite or inhibit the vasomotor centre. Under normal conditions, the vasoconstrictor area of the vasomotor centre transmits signals continuously to the sympathetic vasoconstrictor nerve fibres all over the body called 'sympathetic vasoconstrictor tone' which maintains a partial state of contraction in the blood vessels, called 'vasomotor tone' (Guyton and Hall, 2011).

The sympathetic nervous system plays a significant role in the regulation of vascular tone. NE is the main neurotransmitter of the sympathetic nervous system and is released by the sympathetic postganglionic neurons which terminate in the effector organs including the vascular smooth muscle cells. Here, α-1 adrenergic receptors are activated causing vasoconstriction. NE also has a smaller effect on β-2 adrenergic receptors, which cause vasodilatation; however, the net effect of NE on the vasculature is that of vasoconstriction (Bennett and Gardiner, 1996). The innervations of the small arteries and arterioles (resistance vessels) allows sympathetic stimulation to increase the resistance to blood flow.

1.1.3.1 Non-adrenergic non-cholinergic and nitrergic nerves

ACh and NE are not the only autonomic transmitters. The presence of non-adrenergic non-cholinergic (NANC) transmission includes many potential neurotransmitters including adenosine triphosphate (ATP), vasoactive intestinal peptide (VIP), neuropeptide Y (NPY), substance P, 5-hydroxytryptamine, gamma-aminobutyric acid (GABA), dopamine and NO (Lundberg, 1996). The NANC autonomic nerves are widely distributed including in the gastrointestinal, respiratory and urogenital tracts, as well as vascular smooth muscle.

The discovery of NO as a neurotransmitter was a major breakthrough in understanding the mechanisms underlying NANC neurotransmission (Toda and Okamura, 2003). Nerves in which the transmission process utilises the L-arginine-NO pathway are termed 'nitrergic' nerves (Rand, 1992) and vascular smooth muscle is innervated by neurons containing neuronal nitric oxide synthase (nNOS) (Bredt et al., 1990; Toda and Okamura, 2003).

1.2 NITRIC OXIDE AND VASCULAR TONE

Furchgott and Zawadski showed that vascular endothelial cells must be present for acetylcholine to induce relaxation of rabbit aorta (Furchgott and Zawadzki, 1980). The endogenous mediator causing endothelium dependant relaxation was initially named endothelium derived relaxing factor (EDRF), and was later identified as NO (Ignarro et al., 1987; Palmer et al., 1987). NO is a major modulator of vascular tone. Its prime function is to counterbalance the vasoconstrictive influence of the sympathetic nervous

and renin-angiotensin systems. In conjunction with other vasodilators such as PGI_2 and EDHF, NO acts directly on vascular smooth muscle cells to induce muscle relaxation (Davignon and Ganz, 2004; Feletou and Vanhoutte, 1999; Jeremy et al., 1999; Kharbanda and Deanfield, 2001; Li and Forstermann, 2000; Vallance and Chan, 2001; Verma and Anderson, 2002).

The stimulus for a continuous production of NO includes the blood flowing through a vessel, which exerts a frictional force on the luminal surface of the endothelium (shear stress), and agonists, factors that increase intracellular calcium including local mediators and neurotransmitters (Guyton and Hall, 2011).

1.2.1 Nitric oxide synthesis

NO is a highly labile molecule synthesized from L-arginine and molecular oxygen, by the nitric oxide synthase (NOS) group of enzymes (Li and Forstermann, 2000; Michel and Feron, 1997; Moncada and Higgs, 1993). This process requires a number of co-factors such as nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide, flavin adenine dinucleotide, tetrahydrobiopterin (BH4) and calmodulin. NO diffuses across the endothelial cell membrane into the vascular wall and/or vascular lumen where it exerts its physiological effects.

NO acts on its target cells (such as vascular smooth muscle cells and platelets) by activating the enzyme guanylate cyclase, and in turn increasing the concentration of the intra-cellular messenger cyclic guanosine-3’5-monophosphate (cGMP) (Moncada and Higgs, 1993). cGMP mediates many of the effects of NO such as reduction in

vascular tone and platelet activation. In addition, NO can directly bind other targets such as haem and other iron centred proteins, deoxyribonucleic acid (DNA), thiols and enzymes of the mitochondrial chain altering mitochondrial respiration in tissues

1.2.2 Nitric oxide synthases

NO is generated by three different isoforms of NOS. NOS I, or neuronal NOS (nNOS) was first identified in central and peripheral neuronal cells, but is also found in other tissues such as skeletal (Kobzik et al., 1994) and cardiac (Xu et al., 1999) muscle. It plays an important role in controlling neuronal activity. NOS II, or inducible NOS (iNOS) can be expressed in many different cells. Inflammation induces production of iNOS, which in turn helps to propagate the inflammatory process. NOS III, or endothelial NOS (eNOS) was first identified in endothelial cells where it plays a pivotal role in the homeostatic function of the endothelium (Fleming and Busse, 1999; Li and Forstermann, 2000; Michel and Feron, 1997; Shah et al., 1999) and is also expressed in cardiomyocytes, platelets, hippocampus neurons, renal epithelial cells and mast cells (Michel and Feron, 1997). In humans, the genes encoding eNOS, nNOS and iNOS are located on chromosome 7, 12 and 17 respectively. eNOS and nNOS are constituents of healthy cells, however iNOS is generally expressed in response to inflammation and infection.

1.2.3 eNOS and vascular tone

A large body of data from animal and human studies indicate that eNOS plays a pivotal role in the regulation of vascular tone. The most definitive evidence is derived

from eNOS null mice, where vasodilatation elicited by increases in flow or agonists such as acetylcholine and substance P is impaired (Moncada and Higgs, 1993; Moncada and Higgs, 2006). In humans, local intra-arterial infusion of the non-selective NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA) inhibits vasodilatation in response to acetylcholine infusion or shear stress due to increased blood flow, supporting a role of eNOS in regulation of vascular tone (Joannides et al., 1995; Moncada and Higgs, 1993; Moncada and Higgs, 2006; Quyyumi et al., 1995a).

1.2.4 NO and basal vascular tone

In addition, there is evidence that continuous release of NO may be responsible for basal regulation of vascular tone, known as 'tonic vasodilatation'. Studies by Vallance and colleagues in healthy humans demonstrated that local infusion of L-NMMA into the forearm significantly reduced resting blood flow. This effect was abolished by L-arginine but not D-arginine, thereby confirming that locally generated NO regulates basal microvascular tone (Vallance et al., 1989). Similar findings have been demonstrated in other vascular beds *in vivo*, for example, in the coronary circulation (Lefroy et al., 1993; Quyyumi et al., 1995a). Further indirect evidence supporting the role of NO in tonic vasodilatation is derived from the observation that infusion of L-NMMA leads to dose-dependent hypertension in animals (Rees et al., 1989) and humans (Haynes et al., 1993). The effects of a non-isoform selective NOS inhibitor on basal vascular function or blood pressure have generally been attributed to continuous release of NO from eNOS. However, they do not conclusively prove its involvement since L-NMMA inhibits all NOS isoforms.

1.2.5 Endothelial dysfunction

Endothelial cell dysfunction is a broad term used to describe a state of *endothelial activation*, which is characterised by a reduction in the bioavailability of endothelial vasodilators and creation of a pro-inflammatory and pro-coagulant vascular milieu (Behrendt and Ganz, 2002; Davignon and Ganz, 2004; Landmesser et al., 2004; Verma and Anderson, 2002). Changes in the intricate balance between endothelium-derived factors with opposing properties, in particular a reduction in the bioavailability of NO, forms the basis of endothelial dysfunction. Endothelial dysfunction is a key step in the initiation and progression of atherosclerosis.

Endothelial dysfunction is often assessed indirectly in terms of the extent of endothelium-dependent vasodilatation in response to pharmacological, physical and/or physiological stimuli, based on the assumption that impaired endothelial vasomotion also reflects alterations in other critical properties of the endothelium. The rationale for this approach is dependent on the observation that NO, a key determinant of endothelial vasomotor function, is also critically involved in the regulation of other homeostatic properties of the endothelium. Numerous studies have demonstrated that the presence of coronary atherosclerosis is associated with impaired NO-mediated vasomotor responses (Cox et al., 1989; Gordon et al., 1989; Ludmer et al., 1986; Quyyumi et al., 1995b; Zeiher et al., 1991) and that endothelial dysfunction occurs very early in the disease process. Many studies have demonstrated coronary or peripheral vasomotor dysfunction in patients with risk factors for atherosclerosis who have not yet developed clinically overt disease (Quyyumi et al., 1995b; Quyyumi et al., 1997; Vita et al., 1990) and that the degree of impairment relates to the number of

risk factors present (Egashira et al., 1993; Vita et al., 1990). Endothelial dysfunction is itself independently related to future adverse cardiovascular events (Gonzalez and Selwyn, 2003).

1.3 nNOS AND VASCULAR TONE

1.3.1 Introduction to nNOS

nNOS has been found in a variety of sites including brain, spinal cord, sympathetic ganglia, adrenal glands, peripheral nitrergic nerves, epithelial cells of various organs, kidney macula densa, pancreatic islet cells, vascular smooth muscle cells (Forstermann et al., 1994), cardiac myocytes (Xu et al., 1999) and skeletal muscle (Kobzik et al., 1994). It has been implicated in modulating learning, memory and neurogenesis (Zhou and Zhu, 2009). In the central nervous system (CNS), nNOS mediates long-term regulation of synaptic transmission (O'Dell et al., 1991; Schuman and Madison, 1994) and there is evidence that inhibitors of nNOS impair learning and result in amnesia in animals (Bohme et al., 1993; Holscher and Rose, 1992).

There is evidence that nNOS-derived NO in the CNS is involved in the central regulation of blood pressure (Toda et al., 2009) and increasingly that peripherally nNOS is involved in the local regulation of vascular tone. The conventional view of NO-dependent regulation of blood flow in humans has been that this mainly involves eNOS in the endothelium of blood vessels. An emerging literature from animal and human studies suggests that nNOS-derived NO may also exert local vascular effects.

1.3.2 Animal evidence for local nNOS-mediated regulation of vascular tone

nNOS is known to be expressed in the peripheral nervous system (Toda and Okamura, 2003), primarily in NANC (or nitrergic) nerves, as well as in skeletal (Kobzik et al., 1994) and cardiac (Xu et al., 1999) muscle and the vessel wall (Kavdia and Popel, 2004). Animal studies indicate that non-neurological nNOS has important effects on the regulation of vascular tone.

Ichihara and colleagues provided direct evidence for nNOS mediated regulation of vascular tone in explanted rat kidneys. The nNOS-selective inhibitor, S-methyl-L-thiocitrulline (SMTC), decreased basal diameter of afferent and efferent arterioles but had no effect on vasodilatation to ACh (an eNOS agonist), suggesting that basal tone in this bed was regulated by nNOS-derived NO (Ichihara et al., 1998). The cells of the macula densa are thought to be the main source of renal nNOS (Wilcox and Welch, 1998), and when these were removed, the effects of SMTC were abolished (Ichihara et al., 1998). Some *in vivo* studies in rats have reported that acute nNOS inhibition with SMTC increases blood pressure (Gozal et al., 1996a; Komers et al., 2000). In nNOS knockout mice, the regulatory influence of the macula densa in the kidney was significantly attenuated (Vallon et al., 2001). Some *in vivo* studies in rats have reported that acute nNOS inhibition with SMTC increases blood pressure (Gozal et al., 1996a; Komers et al., 2000). Another nNOS-selective inhibitor, vinyl-L-N^5-(1-imino-3-butenyl)-L-ornithine (L-VNIO), similarly reduced basal tone in rat mesenteric arteries without endothelium (Hatanaka et al., 2006). L-VNIO increased the vasoconstrictor response to perivascular nerve stimulation and local NE concentration, without influencing the effect of endogenous NE, suggesting that nNOS-derived NO affects

neurotransmitter release from the perivascular nerves (Hatanaka et al., 2006). Selective nNOS inhibition with *N*-(4S)-(4-amino-5-[aminoethyl] aminopentyl)-*N*-nitroguanidine (N4S) also reduced *in vivo* cerebral arteriolar diameter in rats as well as attenuating cerebral hypoxia-induced vasodilatation (Bauser-Heaton and Bohlen, 2007).

1.3.3 Indirect human evidence for local nNOS-mediated regulation of vascular tone

It has been well documented that nNOS-derived NO is the predominant vasodilator in penile erectile function (Burnett et al., 1993), and a reduction in NO availability is thought to contribute to erectile dysfunction. Phosphodiesterase type 5 (PDE5) inhibitors, such as sildenafil, are extremely efficacious in restoring the nNOS-cGMP mediated vasodilatation in erectile tissue (Ghofrani et al., 2006). There has been an association between erectile dysfunction and hypertension, along with other cardiovascular risk factors, and these have been attributed to decreased availability of NO in both erectile tissue and the vasculature (Solomon et al., 2003). This raises the possibility that nNOS-derived NO is reduced in the peripheral vasculature in hypertension. It is also worth noting that sildenafil is effective in lowering blood pressure (Oliver et al., 2006).

Abundant nNOS has also been demonstrated in the adventitial nerves of saphenous vein grafts harvested for coronary bypass surgery (Tsui et al., 2002), as well as the human internal mammary artery, where it was predominantly found in the smooth muscle (Webb et al., 2006).

1.3.4 Human evidence for local nNOS-mediated regulation of vascular tone

Table 1.1 outlines some of the nNOS-selective inhibitors used in animal studies (Babu and Griffith, 1998; Cottyn et al., 2008; Furfine et al., 1994; Handy et al., 1996). Due to a lack of safe and clinically validated nNOS-selective inhibitors, more direct investigation of the role of nNOS in the regulation of vascular tone in humans *in vivo* has not been possible until recently. Using the nNOS-selective inhibitor SMTC, our lab recently undertook a series of first-in-human studies to investigate the possible role of nNOS in both the peripheral and coronary circulation (Seddon et al., 2009; Seddon et al., 2008). SMTC has a 17-fold selectivity for inhibition of nNOS over eNOS and is a more potent inhibitor of nNOS than L-NMMA with relative IC_{50} values of 0.31 μM and 4.1 μM respectively (Furfine et al., 1994). SMTC was chosen because (a) it had been extensively used *in vivo* in animal studies where it was found to be safe (Cervenka et al., 2001; Damy et al., 2003; Ichihara et al., 1998; Komers et al., 2000; Narayanan et al., 1995; Wakefield et al., 2003), (b) it is water-soluble (Furfine et al., 1994; Narayanan et al., 1995), (c) it is a reversible inhibitor (Furfine et al., 1994; Narayanan et al., 1995), and (d) animal studies had established concentrations at which it is nNOS-selective *in* vivo (Furfine et al., 1994).

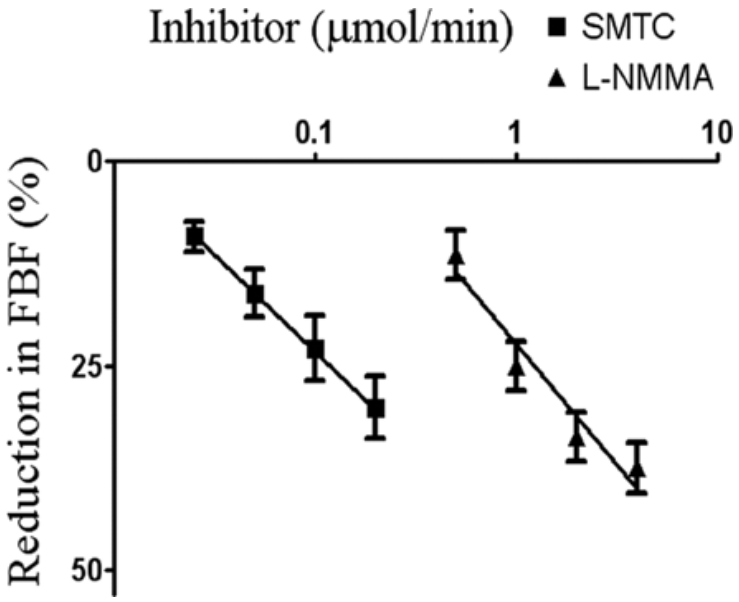
Table 1.1 Neuronal NOS inhibitors used in animal studies.

INHIBITOR	MODE OF ACTION	SELECTIVITY
L-VNIO Vinyl-L-N^5-(1-imino-3-butenyl)-L-ornithine	Damage to oxygenase domain, loss of the heme cofactor (irreversible)	120 fold selectivity for nNOS compared to eNOS *in vitro*
7-NI 7-Nitroindazole	Binding to haem group, disrupting electron flow, interfering with the binding of L-arginine and BH_4 (reversible)	10 fold selectivity over iNOS *in vivo*. No selectivity *in vitro*
TRIM 1-(2-trifluoro-methyl-phenyl) imidazole	Binding to haem group, disrupting electron flow, interfering with the binding of L-arginine and BH_4 (reversible)	37 fold selectivity for nNOS compared to eNOS *in vitro*
SMTC S-methyl-L-thiocitrulline	Binding to heme moiety, preventing subsequent oxygen activation (reversible)	17 fold selectivity for nNOS compared to eNOS *in vivo*

1.3.4.1 Forearm vasomotor tone

Local infusion of SMTC into the brachial artery of healthy subjects resulted in a significant dose-dependent reduction in basal forearm blood flow (FBF).The effect was abolished in the presence of excess L-arginine but not D-arginine, indicating that the effects were mediated by stereospecific inhibition of the L-arginine/ NO pathway (Seddon et al., 2008). SMTC reduced basal FBF at substantially lower (over 10-fold) concentrations than L-NMMA, consistent with its greater potency as a nNOS-selective inhibitor.

Figure 1.1: Effects of SMTC and L-NMMA on basal blood flow. Comparative dose responses to SMTC and L-NMMA (Seddon et al., 2008).



SMTC had no effect on increases in FBF elicited by infusion of acetylcholine or on FMD induced by reactive hyperaemia, which were however inhibited by L-NMMA (Seddon et al., 2009; Seddon et al., 2008). Taken together, these results indicate that basal microvascular tone and blood flow in the human forearm vascular bed is regulated mainly via local nNOS-derived NO whereas increases in flow stimulated by acetylcholine and FMD are eNOS-mediated. Since L-NMMA is non-selective for nNOS and eNOS, it is able to reduce both basal FBF and the stimulated response to acetylcholine or shear stress whereas SMTC only affects the former.

1.3.4.2 Coronary vasomotor tone

This data was significantly extended in further studies in which the effects of SMTC in the human coronary circulation were investigated (Seddon et al., 2009). Peripheral and myocardial circulations differ in terms of microvascular architecture, the pattern of blood flow and vascular resistance, metabolic regulation and the pathways that are activated to induce hyperaemia (Komaru et al., 2000). Despite this, peripheral artery vasomotor function of individuals has been shown to be closely related to vasomotor function in their coronary arteries (Anderson et al., 1995). In patients with angiographically normal coronary arteries, the intra-coronary infusion of SMTC significantly reduced basal blood flow but had no effect on increases in flow induced by infusion of substance P (an eNOS agonist). In contrast, substance P-induced increases in coronary flow were inhibited by L-NMMA (Seddon et al., 2009). These data indicate that local nNOS-derived NO is also a regulator of basal blood flow in the human coronary circulation.

1.3.4.3 Mental stress response

NO has been shown to play a role in the increase in FBF induced by mental stress. Intra-brachial non-selective NOS inhibition with L-NMMA attenuated the increase in FBF produced by standardised mental stress protocols (Cardillo et al., 1997; Dietz et al., 1994). Seddon and colleagues found that this vasodilator response was attenuated by both L-NMMA and SMTC, but not by norepinephrine, which reduced basal FBF to a similar extent to both NOS inhibitors (Seddon et al., 2008). These results suggest a

role for local nNOS-derived NO in the regulation of mental-stress induced vasodilatation.

1.3.4.4 Skin vasculature

Further evidence for a role of nNOS in regulating human vascular tone comes from Kellogg and colleagues. These authors have shown in a series of studies that nNOS and eNOS have distinct roles in the regulation of human cutaneous vasculature *in vivo*. Cutaneous vasodilation in the forearm of healthy volunteers in response to local skin warming was attenuated by intra-dermal administration of the eNOS-selective inhibitor N^G-amino-L-arginine (L-NAA), but not the nNOS-selective inhibitor N^W-propyl-L-arginine (N-PLA). Combined inhibition of both isoforms with LNAA and NPLA had the same effect as with L-NAA alone. In contrast, cutaneous vasodilatation in response to whole body heat stress was attenuated by N-PLA but not L-NAA, with combined inhibition having similar effect as N-PLA alone (Kellogg et al., 2009). This was in keeping with their previous experiments in which cutaneous vasodilation caused by local skin warming was inhibited by the non-selective NOS inhibitor, N-nitro-L-arginine methyl ester (L-NAME) (Kellogg et al., 1999), and also by L-NAA (Kellogg et al., 2008a), and cutaneous vasodilation due to whole body heat was attenuated in the presence of the nNOS-selective inhibitor 7-nitroindazole (7-NI), but not during local skin warming (Kellogg et al., 2008b). They concluded that local nNOS is responsible for centrally mediated, reflex cutaneous active vasodilatation in whole body heat stress, whereas eNOS mediates the increase in skin blood flow in response to axon reflex mediated local warming of the skin.

1.4 PHYSIOLOGICAL ROLES OF LOCAL nNOS IN VASCULAR REGULATION

Short-term adjustments in vessel tone mediated through an interplay between autonomic activity and local regulatory factors are essential in ensuring that blood flow distribution among different vascular beds occurs in line with varying metabolic demands to achieve optimal perfusion and function (Harris and Matthews, 2004; Joyner and Dietz, 2003). This interaction is especially important during situations where vascular regulation is important, such as exercise, when an increase in blood flow to the exercising skeletal muscle as well as to myocardium is essential for normal function. It is of interest to consider whether nNOS-derived NO may be involved.

1.4.1 Blood flow regulation in exercising skeletal muscle

An important mechanism that contributes to the increase in blood flow in exercising skeletal muscle is the local attenuation of sympathetic vasoconstriction – a phenomenon known as 'functional sympatholysis' (Remensnyder et al., 1962). There is increasing evidence from experimental animal studies and some indirect human data that nNOS-derived NO may contribute to this process.

1.4.1.1 Animal evidence suggesting a role for nNOS-derived NO in functional sympatholysis

Many animal studies suggest a potential role for NO, and more specifically nNOS-derived NO in regulating blood flow by blunting the vasoconstrictor response to α-

adrenergic activation during dynamic exercise (functional sympatholysis) and/or contributing to exercise induced vasodilation. In the rat hind limb, non-selective NOS inhibition with L-NAME substantially increased muscle vasoconstriction in response to lumbar sympathetic nerve stimulation (Thomas and Victor, 1998), suggesting that sympathetic nerve activity may stimulate NO release from nNOS and/or eNOS A similar response has been shown in a dog hind limb model of exercise (Buckwalter et al., 2004) and contracting mouse muscle in which nNOS was absent (Fadel et al., 2003; Grange et al., 2001). Moreover, in nNOS null mice and in the *mdx* mouse, an animal model of Duchenne Muscular Dystrophy (DMD) where dystrophin deficiency results in greatly reduced nNOS expression in skeletal muscle, the normal ability of muscle contraction to attenuate α-adrenergic vasoconstriction was defective and led to abnormal flow regulation (Thomas et al., 1998). Lai and colleagues also showed that skeletal muscle perfusion was compromised in the *mdx* mouse. They used transgenic *mdx* mice (a model of DMD with transplanted nNOS anchoring proteins) and showed that they have greater active locomotor muscle blood flow and exercise performance compared to the non-transgenic *mdx* counterparts in which nNOS is absent (Lai et al., 2009).

Kobayashi and colleagues have suggested that nNOS in skeletal muscle contributes to increased blood flow after mild exercise in mouse models (Kobayashi et al., 2008). They showed that nNOS null mice have an exaggerated fatigue response to mild exercise. In mouse models of nNOS mislocalization from the sarcolemma (such as the *mdx* mouse), prolonged inactivity was only relieved by pharmacologically enhancing the cGMP signal that results from muscle nNOS activation during the NO-signalling response to mild exercise (Kobayashi et al., 2008). Percival and colleagues reported

moderate contraction-induced fatigue and reduced post exercise strength in nNOSμ-deficient muscle. They reported that they have demonstrated 2 functionally distinct nNOS micro-domains in skeletal muscle. Sarcolemmal nNOSμ matches blood supply to the metabolic demands of the active muscle and that nNOSβ simultaneously modulates the ability of skeletal muscle to maintain force production during and after exercise (Percival et al., 2008; Percival et al., 2010)

Taken together, the experimental data above suggests that nNOS-derived NO plays a major role in flow regulation during exercise, at least in part through functional sympatholysis.

1.4.1.2 Animal evidence which does not suggest a major role for nNOS-derived NO in functional sympatholysis

Copp and colleagues used nNOS-selective inhibiton with SMTC at rest and sub-maximal exercise in healthy rats. This experiment was the first of its kind to investigate systematically the effects of selective nNOS inhibition on muscle blood flow at rest and exercise in the conscious rat. They found a reduction in baseline hindlimb muscle blood flow, but total hindlimb muscle blood flow during exercise was not different between control and SMTC (Copp et al., 2010).

1.4.1.3 Human studies suggesting a major role for NO in functional sympatholysis

Kneale and colleagues locally infused the β_2-adrenergic agonist, albuterol, into healthy humans, which produced an increase in FBF. This effect was attenuated with co-

infusion of L-NMMA (Kneale et al., 2000), suggesting that sympathetic nerve activity may stimulate NO release from nNOS and/or eNOS in humans. However, the role of NO in the regulation of flow in skeletal muscle during exercise in humans remains conflicting. In healthy humans, a number of studies have shown a reduction in forearm blood flow during or immediately after exercise in response to non-selective NOS inhibition with L-NMMA or L-NAME. Gilligan and colleagues found that L-NMMA reduced FBF at rest and during hand-grip exercise in healthy volunteers (Gilligan et al., 1994). Chavoshan and colleagues demonstrated that systemic NOS inhibition with L-NAME completely abolished the blunting of vasoconstrictor responses in contracting forearm muscle during increased sympathetic activity induced by lower body negative pressure (LBNP), as measured by changes in muscle oxygenation by near infra-red spectroscopy (NIRS) (Chavoshan et al., 2002). In a recent study, Wray and colleagues used Doppler ultrasound to examine FBF at rest and during hand-grip exercise in healthy volunteers, and found that L-NMMA attenuated FBF as well as brachial artery vasodilatation during exercise (Wray et al., 2011).

1.4.1.4 Human studies not suggesting a major role for NO in functional sympatholysis

Some studies in healthy humans have not been consistent in demonstrating a role for NO in functional sympatholysis. Wilson and Kapoor (Wilson and Kapoor, 1993) used venous occlusion plethysmography to measure FBF during wrist flexion exercise with or without concurrent infusions of NE to provide α-adrenergic vasoconstriction, and the effect of locally infused L-NMMA (infused prior to exercise) on these responses was determined. L-NMMA significantly reduced resting FBF and blunted the response to ACh, but had no effect on blood flow responses during exercise. Endo and

colleagues found that L-NMMA reduced FBF (measured by venous occlusion plethysmography) at rest and immediately after hand-grip exercise, however the percentage increase of FBF from rest were similar before and after L-NMMA, and the effect of L-NMMA was comparable to a control vasoconstrictor, angiotensin II (Endo et al., 1994). Gordon and colleagues also used L-NMMA in healthy volunteers and found that wrist-flexion induced increase in FBF was attenuated. However, stabilization of resting vasodilator tone by nitroprusside eliminated the effects of L-NMMA on peak flow after exercise (Gordon et al., 2002).

Dinenno and Joyner examined the effects of local NOS inhibition with L-NMMA or L-NAME on FBF responses (using Doppler ultrasound) during handgrip exercise and whilst stimulating release of endogenous norepinephrine (by intra-arterial infusion of tyramine). Neither NOS inhibitor restored the vasoconstrictor response to local tyramine-stimulated norepinephrine release during exercise, arguing against a role for NO in functional sympatholysis (Dinenno and Joyner, 2003). Consistent with these findings, the same group have demonstrated that local infusion of sodium nitroprusside (SNP) (a NO donor) into the brachial artery at doses sufficient to increase FBF to levels observed during exercise does not blunt sympathetic vasoconstriction, induced again via local infusion of tyramine (Tschakovsky et al., 2002).

1.4.1.5 Indirect human studies suggesting a major role for nNOS-derived NO in functional sympatholysis

Direct studies specifically investigating the role of nNOS in the regulation of skeletal muscle blood flow during exercise have not been possible due to the lack of nNOS-

selective inhibitors for human use. However, a study in children with Duchenne Muscular Dystrophy (DMD) who have impaired nNOS-dependent signalling in the skeletal muscle, provides indirect evidence to support a possible role of nNOS in mediating "functional sympatholysis" in humans. In this study, the vasoconstrictor response to LBNP (measured as a decrease in both resting forearm vascular resistance measured by venous occlusion plethysmography, and tissue oxygenation measured by NIRS was blunted during hand-grip isometric handgrip exercise in healthy subjects and in children with limb girdle dystrophy (a muscle disorder with plentiful nNOS), but not in those with DMD (Sander et al., 2000). The authors attributed these effects to the loss of skeletal muscle nNOS-derived NO in children with DMD.

Taken together, the experimental and clinical studies to date potentially suggest an important role for increased NO production in ensuring appropriate perfusion to skeletal muscle during exercise. The recent data on local nNOS-mediated effects on resting blood flow in humans (Seddon et al., 2009; Seddon et al., 2008) together with the aforementioned studies in nNOS deficient mice and children with DMD suggest nNOS-derived NO may be important in mediating the regulation of blood flow to exercising muscle. Human studies using non-selective NOS inhibitors have had conflicting results, but have not been performed in the presence of the increased sympathetic activation expected during systemic exercise.

1.4.2 Coronary vasomotor tone and increased myocardial demand

Now to consider myocardial as opposed to skeletal blood flow. Exercise is the most important physiological stimulus for increased myocardial oxygen demand. The

requirement of exercising muscle for increased blood flow necessitates an increase in cardiac output that results in increases in the three main determinants of myocardial oxygen demand: heart rate, myocardial contractility, and ventricular work. The contribution of the increase in heart rate to the increase in myocardial oxygen consumption may account for 50-70% of the increase in myocardial oxygen consumption during exercise (Gorman et al., 2000; Heiss et al., 1976; Merkus et al., 2004). Increased myocardial oxygen demands during dynamic exercise are met principally by augmenting coronary blood flow (CBF), which increases in proportion to heart rate (Duncker et al., 2012). The strong correlation between coronary flow and heart rate occurs because heart rate is a common multiplier for the other determinants of myocardial oxygen demand (i.e. contractility and cardiac work), which are computed per beat (Duncker and Bache, 2008).

One way to study the relationship between heart rate and coronary blood flow (CBF) is by the use of incremental pacing protocols. It is well established that during cardiac pacing, angiographically normal human coronary arteries dilate and CBF increases, indicating epicardial and microvascular dilatation with increase in myocardial oxygen requirements (Gaglione et al., 1987; Gordon et al., 1989; Nabel et al., 1990a; Quyyumi et al., 1995a). L-NMMA reduces CBF at rest and attenuates ACh-induced increase in CBF (Lefroy et al., 1993; Quyyumi et al., 1995a). Quyyumi and colleagues found in patients with angiographically normal coronary arteries that intra-coronary L-NMMA produced significant basal increase in coronary vascular resistance and a small significant reduction in epicardial coronary artery diameter. Pacing-induced increases in CBF and coronary artery diameter were also attenuated in the presence of L-NMMA (Quyyumi et al., 1995a). Tousoulis and colleagues also found no increase in coronary

artery diameter and significantly attenuated CBF whilst pacing during L-NMMA in angiographically normal arteries (Tousoulis et al., 1997). These data confirm the role of NO in this process. Our lab has previously found that nNOS-derived NO tonically regulates basal CBF whereas eNOS mediates increases in flow in response to the eNOS agonist, substance P (Seddon et al., 2009). However, there is no data on the relative contribution of different NOS isoforms on pacing-induced increases in blood flow in the human coronary circulation.

1.5 nNOS AND BLOOD PRESSURE

The hallmark of primary hypertension is an increase in peripheral vascular resistance, therefore dysfunction of the NO pathway within the peripheral vasculature is an attractive hypothesis as a mechanism contributing to essential hypertension. However there are multiple mechanisms through which NO may regulate blood pressure including central, renal and cardiac, in addition to vascular.

Systemic nNOS inhibition with SMTC has previously been shown to increase blood pressure in rats *in vivo* (Gozal et al., 1996b; Komers et al., 2000; Wakefield et al., 2003), as has the nNOS-selective inhibitor 7-NI (Ollerstam et al., 1997; Xu et al., 2000). However, human studies to date have been restricted to non-selective NOS inhibition.

1.5.1 Human studies using L-NMMA

There are many studies in humans using acute systemic NOS inhibiton with L-NMMA which have consistently found an increase in systemic vascular resistance and blood pressure, with a reduction in heart rate and cardiac output (Brett et al., 1998; Hansen et al., 1994b; Haynes et al., 1993; Stamler et al., 1994). Brett and colleagues infused L-NMMA intravenously in healthy men over 5 minutes. At the maximum dose used of 6 mg/mL there was an increase (when compared with effect of saline placebo) in systolic and diastolic blood pressures of 4.1±1.1 and 12.6±3.5 %, respectively, with a marked increase in systemic vascular resistance index (SVRI) of 39.2±5.2 %. Cardiac index and heart rate were 22.0±3.3 and 17.0±4.4 % lower after administration of L-NMMA (Brett et al., 1998). Stamler and colleagues used invasive measurement of blood pressure and cardiac output (Fick method) in response to 3 mg/Kg of intravenous L-NMMA over 3 min. They found a 15.5±1.3 % increase in mean arterial pressure (MAP) and a 63.4±8.2% in systemic vascular resistance (SVR) (Stamler et al., 1994). The increase in blood pressure is often thought to be a direct effect on vascular tone of basal release of NO, but may involve the central nervous system and renal mechanisms. It has previously been considered that eNOS is the isoform responsible for these effects, but there is increasing evidence that nNOS is also involved in the regulation of blood pressure.

1.5.2 nNOS and the kidney

1.5.2.1 Renin-angiotensin-aldosterone system

The renin-angiotensin-aldosterone system synergises with the sympathetic nervous system, for example by increasing the release of NE from sympathetic nerve terminals. It also stimulates aldosterone secretion and plays a central role in the control of sodium excretion and fluid volume, as well as vascular tone. Renin secretion occurs in response to various physiological stimuli including reduced renal perfusion pressure or reduced sodium concentration. It acts on angiotensinogen to form angiotensin I, which is then converted to angiotensin II by angiotensin converting enzyme (ACE). The main effects of angiotensin II are mediated by acting on AT1 receptors, which belong to the family of G-protein-coupled receptors. Effects mediated by AT1 receptors include generalised vasoconstriction, increased NE release (reinforcing sympathetic effects), proximal tubular reabsorption of sodium, secretion of aldosterone from the adrenal cortex (Guyton and Hall, 2011).

1.5.2.2 NO and the kidney

In the kidney, NO regulates sodium and water homeostasis (Lahera et al., 1991) via numerous mechanisms, and impaired renal NO synthesis is implicated in the pathogenesis of hypertension (Pallone and Mattson, 2002). These mechanisms include regulation of renal and glomerular haemeodynamics (Majid and Navar, 2001), mediation of pressure natriuresis (Majid et al., 1993), maintenance of medullary perfusion (Mattson et al., 1992), inhibition of tubular sodium reabsorption (Ortiz and

Garvin, 2002) and modulation of sympathetic nerve activity (Eppel et al., 2003). There have been a number of reports demonstrating decreased renal blood flow, increased renal vascular resistance and elevated systemic blood pressure after treatment with non-selective NOS inhibitors in rats (Gabbai, 2001; Tost et al., 2000) and dogs (Majid and Navar, 2001). eNOS-derived NO has been recognised to play important roles in controlling renal haemodynamics, including increases in the renal plasma flow and pre and post glomerular arteriolar dilatation (Toda and Okamura, 2011). Impairment of this system leads to kidney dysfunction and systemic hypertension (Zoccali, 2007). In healthy humans, Bech and colleagues found that systemic L-NMMA induced a decrease in renal plasma flow, glomerular filtration rate (GFR) and reduction in tubular reabsorption of sodium (Bech et al., 1996). In another study with healthy volunteers, the use of positron emission tomography (PET) to determine renal blood flow showed that this flow was reduced after systemic infusion of L-NMMA (Damkjaer et al., 2010).

1.5.2.3 nNOS in the kidneys

nNOS has also been found in the kidney, and may also contribute to the regulation of renal blood flow and kidney function (Mount and Power, 2006). The cells of the macula densa are thought to be the main source of renal nNOS (Wilcox and Welch, 1998), and when these were removed, the effects of SMTC were abolished (Ichihara et al., 1998). Ichihara and colleagues delivered intra-arterial SMTC at increasing local doses into the isolated afferent and efferent arterioles of rat kidneys and demonstrated a clear dose-dependent effect of SMTC on basal tone of both afferent and efferent arterioles (Ichihara et al., 1998). Renal flow was decreased with intra-aortic SMTC in

control and diabetic rats at a dose which did not affect blood pressure (Komers et al., 2000). Intra-renal SMTC caused a decrease in the renal plasma flow, GFR and absolute sodium excretion in anaesthetized rats, with no effect on blood pressure (Cervenka et al., 2001). In human tissue, nNOS has been found in the arteriole endothelium and renal nerves containing nNOS were found in perivascular connective tissue and near the pelvic epithelium (Bachmann et al., 1995).

1.5.3 nNOS and the nervous system

NO from nNOS is known to play important roles in the brain, mainly as a neuro-modulator (Kiss and Vizi, 2001; Toda et al., 2009). In the peripheral nerves, NO transmits information from the nerves to the smooth muscle (Toda and Okamura, 1996, 2003). The presence of NANC nerves were discovered in various smooth muscle but it was the revelation that NO acts as a neuro-transmitter that was seen as a great step forward, and this hypothesis is now widely accepted as an important control mechanism in functions of autonomically innervated organs and tissues (Toda and Okamura, 2003). The nerves which depend on the release of NO are called 'nitrergic' (Rand, 1992).

In animal models, NO appears to restrain sympathetic drive in the brain and increased nNOS gene expression in hypertensive animals would counterbalance the increased sympathetic drive responsible for systemic hypertension (Toda et al., 2009). In a variety of mammals, peripheral arteries are innervated by nitrergic nerves that contribute to counteracting vasoconstrictor sympathetic nerve activity (Okamura et al., 1995; Toda and Okamura, 1990). Toda and colleagues found that inhibition with the

non-selective NOS inhibitor N^G-nitro-L-arginine (L-NA) in isolated mesenteric arteries in dogs augmented the constrictor response to nicotine (which stimulates neurotransmitters from nitrergic nerves), without any effect on the response to exogenous norepinephrine, despite the absence of the endothelium (Toda and Okamura, 1990). Similar findings were also found in rat mesenteric arteries (Ferrer et al., 2000). SMTC was also found to produce hypertension in conscious rats (Wakefield et al., 2003), however knockout mice deficient in nNOS were not hypertensive (Huang et al., 1994) but showed behavioural abnormalities suggestive of increased sympathetic drive (Nelson et al., 1995)

The brainstem is the major region responsible for sympathetic regulation of blood pressure. In rats microinjection of the NO donor SNP into the periventricular nucleus decreases blood pressure and heart rate, whereas microinjections of L-NMMA or an nNOS antisense oligonucleotide increased blood pressure (Wang et al., 2005). Reduced nNOS expression in brainstem nuclei appears to be responsible for the enhanced sympathetic drive in heart failure. Expression of nNOS protein and mRNA were reduced in rats with heart failure compared with control (Hirooka et al., 2003). There was also increased nNOS gene expression in the brain of hypertensive rats suggesting an important role of central NO in the modulation of sympathetic activity (Plochocka-Zulinska and Krukoff, 1997). Hypertensive rats also had increased nNOS activity in the hypothalamus and brainstem (Qadri et al., 2003). NO appears to inhibit sympathetic drive in the brain, with an increase in nNOS gene expression in hypertensive animals counteracting the increased sympathetic drive responsible for hypertension.

1.6 HYPOTHESIS AND AIMS

The role of NO is central to the local regulation of vascular tone and is also involved in the regulation of blood pressure. Our entire understanding of the role of NO in vascular function in humans *in vivo* came from the use of L-NMMA, a non-selective NOS inhibitor. Recent studies have directly addressed the role of local nNOS in human vascular function *in vivo* using, and importantly, validating the nNOS-selective inhibitor SMTC. The availability of a nNOS-selective inhibitor suitable for human use has given us a platform to further evaluate the role of nNOS in cardiovascular regulation, not only at rest, but during physiological stimuli, none more important than exercise. This is particularly relevant since an increasing volume of animal literature suggests that nNOS also has important effects on vascular function and blood pressure. SMTC to date has only been administered locally, and whilst its effects suggest that local nNOS-derived NO regulates basal vascular tone, there is no evidence available on the role of nNOS on systemic haemodynamics such as blood pressure in humans *in vivo*.

1.6.1 Hypothesis

The hypotheses underlying this work are that (a) local nNOS-derived NO plays an important physiological role in the regulation of myocardial and skeletal blood flow during exercise, and (b) at a systemic level, nNOS plays an important role in the regulation of blood pressure in humans *in vivo*.

1.6.2 Aims of thesis

1. To investigate the role of nNOS on forearm vasomotor tone during exercise +/- reflex sympathetic activation, using local intra-brachial infusion of SMTC.

2. To investigate the role of nNOS on coronary vasomotor tone during increased cardiac workload induced by atrial pacing, using local intra-coronary infusion of SMTC.

3. To investigate the role of nNOS on systemic haemodynamics in a first-in-man study using intravenous SMTC.

CHAPTER 2:

GENERAL METHODS

2.1 NOS INHIBITORS

2.1.1 SMTC

SMTC is a selective inhibitor of nNOS which has been used safely in a large number of animal studies (Cervenka et al., 2001; Damy et al., 2003; Ichihara et al., 1998; Komers et al., 2000; Narayanan et al., 1995; Wakefield et al., 2003), and it is soluble in both water and blood (Furfine et al., 1994; Narayanann et al., 1995). SMTC is a competitive inhibitor with L-arginine, and inhibition of NOS has been demonstrated to be fully reversible. The structure of SMTC is based on the amino acids L-arginine and L-citrulline, and is very similar in structure and function to L-NMMA, which has been used extensively and safely in human studies *in vivo*. There is no published evidence of nNOS-dependent adverse effects on local vessel thrombosis, platelets, or red blood cells.

Our group had previously obtained ethical approval for first-time-use-in-man for SMTC, granted by both St Thomas' Hospital and King's College Hospital Research Ethics Committees following submission of a comprehensive report to an independent toxicologist incorporating a full review of the literature relating to previous use of SMTC in addition to its pharmaceutical and toxicological profiles (Seddon et al.,

2008). Further ethical approval for current research studies was again granted by St Thomas' Hospital and King's College Hospital Research Ethics Committees.

The raw material was purchased from Calbiochem, a subsidiary of Merck Biosciences, UK. SMTC was packaged by the pharmacy department at Guy's & St. Thomas' Hospitals, in 5 ml vials, at the concentration requested for each study. Every batch of frozen SMTC was made up specifically for each pre-designed study and was given a precautionary one-month expiry date. On each study day, vials of frozen SMTC were thawed and filtered through a microbiological microfilter (PALL Posidyne NEO filter).

2.1.2 L-NMMA

L-NMMA was obtained from Bachem, Switzerland and was stored in the fridge at 4°C. On each study day, appropriate concentrations were prepared using serial dilutions under sterile conditions.

2.2 FOREARM STUDIES

2.2.1 Subject participation

All studies were performed in accordance with the Helsinki guidelines. Healthy male volunteers were recruited for the FBF and radial artery diameter studies from a list of suitable subjects who had participated in previous departmental studies. Potential volunteers expressed their interest via email, after which they were given a more

detailed description of the study over the telephone, and this information was subsequently emailed to them as a formal patient information sheet, which had been approved by the local research ethics committee. They were informed of the procedures, the drugs used and their associated risks. Following this, interested subjects were given the opportunity to ask questions and were invited to the department to give written informed consent to participate in the studies. All forearm studies were carried out in healthy volunteers rather than in patients, and some subjects participated in more than one of the study protocols, which were performed on different days at least one week apart.

A brief clinical history was taken to allow documentation of age, sex, smoking and alcohol habits, and confirmation of healthy volunteer status i.e. no medical conditions. Cardiovascular and respiratory examinations were carried out in all subjects. Blood pressure and heart rate were measured using an oscillometric method (Omron 705CP) and according to established guidelines (O'Brien et al., 2003). Mean systolic blood pressure (SBP) and diastolic blood pressure (DBP) values were calculated and used in data analysis (mean of 3 measurements). Readings were taken in a quiet, calm environment in the supine position, after 20 minutes rest. An appropriately sized cuff was used, with the lower end placed approximately 2.5 cm above the ante-cubital fossa. Blood samples for baseline blood parameters were taken well in advance of the study day to ensure that each subject had “normal” blood tests prior to administration of drugs, including lipid profile, full blood count, urea and electrolytes, liver function and clotting studies.

2.2.2 Brachial artery cannulation

Drugs were infused locally via the brachial artery. Drug doses required to provide local effects are usually much smaller than what is required for a systemic dose. Systemic administration of vaso-active drugs may also lead to activation of central effects including changes in sympathetic output and hormonal responses (Collier and Robinson, 1974). To study the effects of local infusion of drug on FBF and radial artery diameter, a 27 gauge steel needle (Coopers Needle Works, Birmingham, UK) was sealed with dental wax to an epidural catheter (Portex, Hythe, UK). This was connected to a constant rate infusion pump (Baxter Healthcare Ltd, Thetford, UK). The cannula is inserted into the brachial artery and advanced 2-3mm to ensure a stable position, and then secured in place with adhesive dressing tape. Saline vehicle and all vasoactive drugs were infused at 1 ml/min. After completion of the experimental protocol, the needle was removed from the brachial artery and pressure applied over the puncture site. No complications due to arterial cannulation arose during any of the studies.

2.2.3 Venous occlusion plethysmography

Bilateral strain gauge venous occlusion plethysmography was used to assess FBF. This technique has been used widely for over 80 years and works on the principle that by occluding venous outflow from a limb whilst allowing arterial inflow to continue, limb volume increases proportionately to total limb arterial blood flow (Whitney, 1953). Changes in limb volume can be measured using strain gauges and flow derived from these values.

Under resting conditions, flow through the forearm is predominantly through skeletal muscle (50-70% of total), the remainder being flow through the skin (Cooper et al., 1955). In contrast, within the hand, which has relatively little skeletal muscle, most blood flows through the skin circulation where blood flow has different physiology and pharmacology to the forearm (Whitney, 1953). In view of this, during measurement of FBF, the wrist cuffs were inflated to suprasystolic pressure (180mmHg) to exclude the hands from the circulation. A second cuff was placed around the upper arm to achieve venous occlusion. This cuff was inflated rapidly to a pressure above venous pressure but below arterial pressure so as to allow arterial inflow to continue (i.e. 40-50mmHg) in repeated cycles, with inflation periods of 5 to 10 seconds followed by 5 seconds of cuff deflation. During inflation, forearm volume increases linearly. The deflation is essential to allow emptying of the forearm veins before the next measurement period as the relationship between rate of increase in forearm volume and arterial flow will not remain consistent if veins become fully distended (Wilkins and Bradley, 1946). Once venous capacitance is exceeded, the limb pressure increases and blood escapes the limb. The forearms were positioned above the level of the heart with supportive foam pads, to allow adequate venous emptying during this deflation period.

The strain gauge is a flexible elastic silicon tube containing mercury. The gauge is fastened under slight tension around the forearm at maximal circumference, at a distance approximately one third the length of the forearm from the ante-cubital fossa (Hokanson et al., 1975). As blood flow into the arm increases, the gauge is stretched leading to a change in the length of the enclosed mercury column and hence the

electrical resistance. The change in output voltage is recorded using computer software (MacLab 400, AD instruments, Australia) and displayed graphically. Venous occlusion leads to radial expansion of the forearm and the rate of change in forearm circumference is proportional to change in arterial blood flow - this is represented in the formula below (Whitney, 1953):

$$\text{FBF (ml/min/100ml forearm)} = K \times \frac{\text{Increase in forearm circumference (mm/min)}}{\text{Forearm circumference (mm)}}$$

(K = proportionality constant)

FBF measurements were made after subjects had rested in a temperature controlled room (between 24-26 C) to allow arterial pressure to stabilise. Blood flow is also dependant on arterial pressure and therefore studies take place in a relaxed, quiet environment so not to affect arterial blood pressure. During the infusion of vasoactive drugs, blood flow was measured during the final minute of each infusion period. The mean of five measurements was used for analysis. The measurement of FBF by venous occlusion plethysmography requires the arms to be motionless, hence FBF measurements were also made for 1min immediately after the cessation of handgrip exercise with the mean of 5 measurements used for analysis. The within-subject coefficient of variation (WCV) for forearm blood flow responses to exercise is similar to that to vasodilator drugs and is in the range 10 to 15% for measures repeated over 1 week (Walker et al., 2001).

2.2.4 Radial artery ultrasound

High resolution vascular ultrasound was used to measure the diameter of the radial artery. The system incorporates vascular software for 2 dimensional (2D) imaging including edge detection software, colour and spectral Doppler, high frequency vascular transducer and an ECG monitor (each image is captured at the same point in the cardiac cycle). A continuous 3 lead electrocardiogram (ECG) recording is made throughout. Images of the radial artery were acquired every 3 seconds using high resolution B-mode ultrasound with a 7-MHz linear array transducer (Acuson Aspen Advanced Imagegate), attached to a computer. Image quality is optimised by adjusting the depth and gain settings so that the longest possible section of artery is visualised, longitudinally. The subjects right arm radial artery was imaged in these studies. The transducer was held in position using a stereotactic clamp which allows minute adjustments in position to be made during acquisition, and the diameter is measured when the clearest picture of anterior and posterior intimal layers is obtained. Once the protocol is started, the machine settings and the patient's arm position must remain fixed. Images were recorded for 1 min for each measurement of diameter. Scans were analysed offline using Vascular research Tools Analyzer for research software programme (Medical Imaging Applications LLC, Iowa USA). This programme uses automated edge detection algorithms to measure the distance between the anterior and posterior walls of the artery throughout the entire acquisition period. This method gives a highly reproducible measure of radial artery diameter with a WCV in the range 2-4% for measures repeated at intervals of a few days.

2.3 CORONARY STUDIES

2.3.1 Subject participation

Coronary blood flow studies were carried out in patients attending the Department of Cardiology, King's College Hospital, London, for elective cardiac catheterisation. Only patients with angiographically normal coronary arteries were to be included in the study. The patients were attending for clinical reasons, usually referred for the investigation of atypical chest pain, but it was only at the time of cardiac catheterisation that would reveal whether the coronary arteries were angiographically normal or not. Potential patients were found by searching through referral letters and then details of the study were given to the patients well in advance of the procedure including a formal patient information sheet approved by the local research ethics committee. Patients were informed of the procedure, drugs used and the associated risks, and that the study would only be carried out if the diagnostic coronary angiogram confirmed angiographically normal arteries, and then written consent was obtained for the study. Patients with valvular heart disease, left ventricular hypertrophy and left ventricular dysfunction were excluded, as were patients with renal or liver failure, or any other significant concurrent disease or condition which in the opinion of the investigators would make the patient inappropriate for participation in the study. All medications were withheld and all patients abstained from food at least 6 hours before cardiac catheterisation.

2.4 SYSTEMIC EFFECTS OF SMTC

In these studies, SMTC was administered systemically for the first time in humans *in vivo* and the effects on systemic haemodynamics were measured.

2.4.1 Subject participation

The studies were approved by the local research ethics committee (St.Thomas' Hospital). Healthy male volunteers were recruited from a list of suitable subjects who had participated in previous departmental studies. The study was also advertised on the King's College London University intranet. Medical students were specifically not recruited. Potential volunteers expressed their interest via email, after which they were given a more detailed description of the study over the telephone, and a formal patient information sheet was emailed to them, which had already been approved by the local research ethics committee. They were informed of the procedures and their associated risks, and the drugs used. Following this, interested subjects were given the opportunity to ask questions and then invited to the department to give written informed consent to participate in the studies. At this point the subjects were screened using the following inclusion and exclusion criteria:

Initial inclusion criteria:

1. Male
2. Aged 18-45 years
3. Able to provide written informed consent

Exclusion criteria:

1. Participation in a research study within the last month in which a systemically active drug has been administered
2. BMI > 28 kg/m^2 (calculated at screening visit)
3. Alcohol intake > 28 U /week
4. Smoking within last 3 months
5. Recreational drug use within last 3 months
6. Regular use of prescription or 'over the counter' medicines which in the opinion of the investigator would make the volunteer inappropriate for participation in the study.
7. Hyperlipidaemia (total cholesterol > 6 mmol/L on blood sample taken at screening visit)
8. Hypertension (SBP > 140, DBP > 90 on ≥ 2 occasions taken at screening visit)
9. Diabetes mellitus (blood sample on day of screening visit)
10. Any history of cardiovascular disease.
11. Any significant concurrent disease or condition which in the opinion of the investigator would make the volunteer inappropriate for participation in the study.
12. Any significant cardiovascular abnormality or other abnormality detected on examination at screening visit.
13. Any significant abnormality of the ECG carried out at screening visit.
14. Any significant abnormality of the full blood count (FBC), renal or liver profiles (performed at screening visit).

Only subjects who met all the above criteria participated in the study.

2.4.2 Methods overview

SMTC was first infused intravenously in a dose-escalation safety study. The second study was a randomised crossover study with blinded haemodynamic assessment in which either the highest dose of SMTC or saline vehicle were infused on separate occasions. Blood pressure (BP) and heart rate (HR) were measured by an oscillometric method whereas stroke volume (SV) was measured using echocardiography. SVR was calculated from the mean MAP and cardiac output (CO). Brachial FMD was measured as an index of eNOS activity. Further details of the methods and protocol are in chapter 5.

2.4.3 Echocardiography

In the first study, left ventricular (LV) stroke volume was measured using 2D trans-thoracic echocardiography, and in the second study using 3-dimensional (3D) trans-thoracic echocardiography.

2.4.3.1 2D echocardiography

When using 2D echocardiography the SV was calculated as cross-sectional area (CSA) of the left-ventricular outflow tract (LVOT) multiplied by the velocity-time integral (VTI) (Otto 2009):

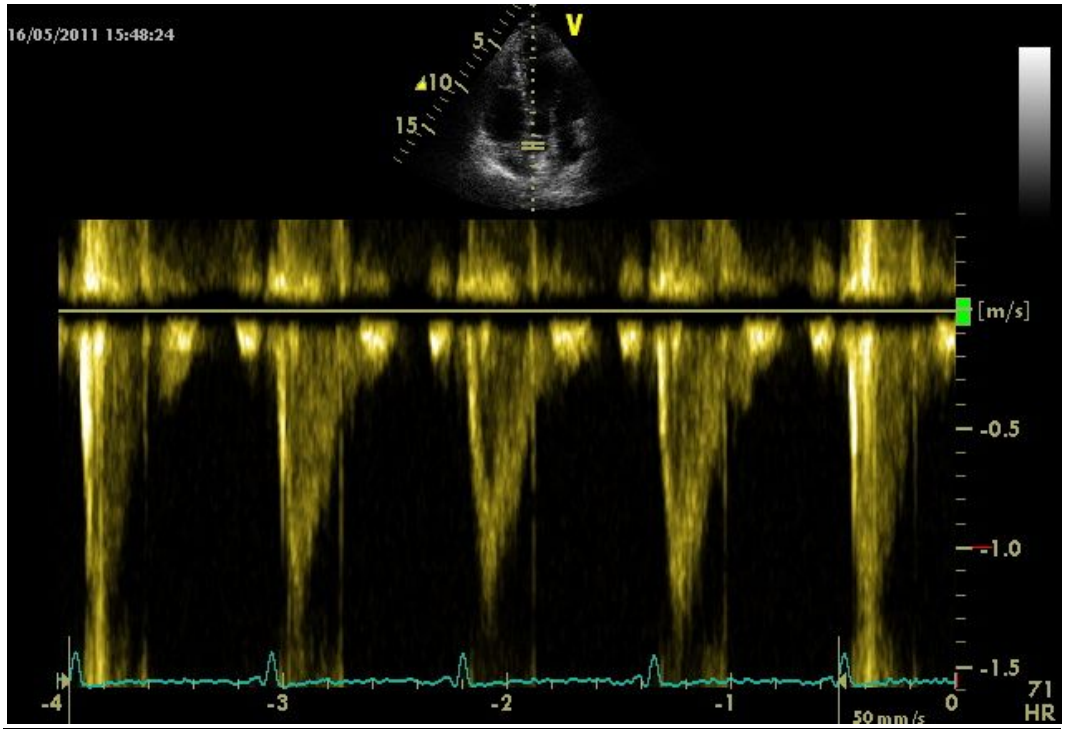
SV = CSA x VTI

This is based on the concept that the left ventricle of the heart pumps blood into the aorta which is cylindrical. The base of the aorta is the systolic CSA of the outflow tract, while its height is the distance blood travels during ejection for each beat. The distance is expressed as the integral of the Doppler systolic velocity-time curve (velocity is the first derivative of distance). The accuracy of this technique depends on accurate CSA measurement. We measured the stroke volume at the LVOT using a para-sternal long-axis view of the heart, diameter (D) being measured parallel and immediately adjacent to the aortic valve, in mid-systole. Area was then calculated using the following equation:

$$\text{Area} = \pi(D/2)^2$$

This is based on the assumption of a circular geometry. Small errors in calculating diameter can lead to larger errors on CSA, and hence these measurements were made twice at baseline and the mean area calculated. The pattern of flow is assumed to be laminar with a spatially flat flow velocity profile. The Doppler signal was recorded at a parallel intercept angle to flow, resulting in an accurate velocity measurement. The Doppler beam can be aligned to obtain the highest velocity signal, which indicates the most parallel alignment with flow. The flow-velocity curve was recorded from an apical 5 chamber view using pulsed wave Doppler ultrasound, using the closing click of the aortic valve to ensure that the sample volume was located at the same site as the diameter measurement.

Figure 2.1: Pulsed Doppler recording of the LV outflow tract from an apical 5 chamber view.



2.4.3.2 3D Echocardiography

Under controlled study settings in patients with adequate image quality, 3D echocardiography offers better accuracy and precision in measuring LV volumes and therefore better precision in measuring LV SV and ejection fraction (EF) compared with 2D echocardiography (Dorosz et al., 2012; Jacobs et al., 2006).

3D echocardiography used to rely upon the acquisition of multiple 2D cross-sectional images, with the spatial and temporal relationship of each image registered and gated to the cardiac and respiratory cycle and then reconstructed to a 3D dataset, which was

time-consuming. This methodology is subject to technical limitations during image acquisition and requires significant offline data processing. The development of real-time 3D echocardiography has eradicated the need for image acquisition protocols and protracted off-line reconstruction. 3D echocardiography uses recently developed matrix array echocardiography probes to image the entire heart and provide improved image quality. LV volume assessment has been demonstrated to be rapid, accurate, reproducible, and superior to conventional 2D methods (Jacobs et al., 2006). A direct volumetric assessment of LV size is preferable to calculations made from 2D because it can guard against image foreshortening and does not require any geometric assumptions. 3D echo assessment of LV volumes and EF also has been shown to compare favourably to the current gold standard of magnetic resonance imaging (MRI) (Jacobs et al., 2006; Jenkins et al., 2004; Sugeng et al., 2006).

Software programs calculate 3D volumes by using semi-automated border tracking techniques to create a mathematical cast of the whole of the LV throughout the cardiac cycle. From this cast the LV volumes (end-diastolic volume, end-systolic volune and SV) and EF are derived. Data analysis was performed offline with dedicated 3D software by experienced operators, Dr Sitara Khan and Dr Benyu Jiang, who were both blinded to all interventions.

2.4.4 Ultrasound for measurement of flow-mediated dilatation

This method is a non-invasive ultrasound-based test to assess conduit artery vascular function and has been used for over 20 years (Celermajer et al., 1992). Conduit artery diameter was measured before and after an increase in shear stress that was induced by

reactive hyperaemia, i.e. FMD, which occurs predominantly as a result of local endothelial release of NO (Joannides et al., 1995). The results are reproducible (Sorensen et al., 1995), especially when carried out by an experienced operator, and correlate with coronary vascular endothelial function (Anderson et al., 1995; Corretti et al., 2002).

Images of the radial or brachial artery are acquired by ultrasound as decribed earlier in this chapter. Subjects right arm radial/brachial artery was imaged longitudinally. A blood pressure cuff was positioned 5-10 cm distal to the transducer on the forearm. The transducer is held in position using a stereotactic clamp which allows minute adjustments in position to be made during acquisition. A baseline image was recorded for 1 min and then the cuff was inflated to at least 50 mmHg above systolic pressure for 5 min inducing ischaemia by restricting blood flow. Release of the cuff leads to a significant increase in conduit arterial flow to accommodate the dilated resistance vessels and this in turn leads to conduit arterial dilatation. Upon cuff deflation the resulting reactive hyperaemia was recorded continuously for 3 min. FMD is taken as the maximal change in dilatation from baseline. Scans are analysed offline as described earlier in this chapter. All artery scanning and FMD analysis was carried out by an experienced operator, Dr Benyu Jiang, who was blinded to all interventions. The WCV of FMD measures repeated at 1 week is in the range 10 to 15% (Donald et al., 2008).

2.5 STATISTICAL ANALYSIS

Data are summarised as mean ± standard error of the mean (s.e.m.) throughout this thesis. Effects of NOS inhibitors on forearm and coronary blood flow and conduit artery diameter, and on haemodynamic responses were analysed by One-way analysis of variance (ANOVA) or by ANOVA for repeated measures as appropriate. All tests were two-tailed and differences were considered significant when $P<0.05$.

Sample sizes for the forearm and coronary protocols are based on the changes in blood flow observed in first-in-man studies using SMTC (Seddon et al., 2009; Seddon et al., 2008). Forearm studies: The within-subject standard deviation of the blood flow responses to exercise is approximately 15%. Therefore, we estimate 16 subjects will give a > 90% power ($P < 0.05$) to detect a change in blood flow response of > 10-15%. Coronary studies: As for the forearm studies, blood flow response within a single study are reproducible (SD < 15%). Therefore, we estimate 16 subjects will give a > 90% power ($P < 0.05$) to detect a change in blood flow response of > 10-15%.

CHAPTER 3:

THE ROLE OF nNOS AND eNOS IN THE REGULATION OF VASOMOTOR TONE DURING FOREARM EXERCISE AND REFLEX SYMPATHETIC ACTIVATION

3.1. INTRODUCTION

NO plays an important role in regulating arteriolar tone, peripheral resistance and blood flow, acting as a tonic dilator opposing sympathetically mediated vasoconstriction (Vallance et al., 1989). In humans, brachial artery infusion of the selective nNOS inhibitor, SMTC, reduces basal FBF but does not block the eNOS-mediated vasodilator response to ACh or increased shear stress (Seddon et al., 2009; Seddon et al., 2008). Animal studies and indirect observations in humans suggest that sympathetic nerve activity may stimulate NO release from eNOS and/or nNOS (Kneale et al., 2000; Thomas and Victor, 1998) implying that local NO release may counteract reflex sympathetic vasoconstriction or contribute to "functional sympatholysis", i.e., the local attenuation of sympathetic vasoconstriction in the exercising muscle. A role for NOS and particularly nNOS in functional sympatholysis is supported by some (Buckwalter et al., 2004; Kobayashi et al., 2008; Thomas et al., 1998) but not all (Copp et al., 2010) animal studies. Human studies using non-specific inhibitors of NOS have not been consistent, but have not been performed in the

presence of the increased sympathetic activation expected during systemic exercise. The recent data on local nNOS-mediated effects on blood flow in humans together with previous studies in nNOS deficient mice and in DMD suggest nNOS- derived NO may be important in mediating the regulation of blood flow to exercising muscle in humans.

We tested the hypothesis that NO release from nNOS and/or eNOS regulated local FBF during reflex sympathetic activation at rest and during exercise. The rationale for the design of the following study was based on Sander and colleagues' study in children with DMD (Sander et al., 2000) in which both the lower body negative pressure (LBNP)-induced decrease in resting forearm vascular conductance and the decrease in tissue oxygenation during hand grip exercise were attenuated in controls, but not in DMD. The authors attributed these effects to the loss of skeletal muscle nNOS-derived NO in children with DMD.

3.1.1 Study aims

The aim of the present study was to use non-selective and selective NOS inhibitors to determine the role of eNOS-derived and nNOS-derived NO in opposing an increase in sympathetically mediated increases in arteriolar tone in the human forearm during handgrip exercise +/- LBNP. The hypothesis underlying this work is that local nNOS-derived NO plays an important physiological role in matching the amount of muscle blood flow to the level of exercise. It is further postulated that this effect may at least in part involve the NO-dependent attenuation of sympathetic vasoconstriction during exercise.

3.2 METHODS

The studies were approved by the local research ethics committee (St.Thomas' Hospital), and all participants provided written informed consent. Healthy male volunteers were recruited by local advertisement. Subjects on regular medication, with hypertension, hyperlipidaemia or any significant abnormality on haematological or biochemical screening were excluded. Participants were asked to abstain from caffeine for at least 12 hours before the studies. The doses of SMTC (0.2μmol/min) and L-NMMA (2μmol/min) used have been shown to inhibit nNOS and both nNOS and eNOS respectively, both at rest and in conditions of increased flow (caused by mental stress and by ACh infusion) (Seddon et al., 2008).

FBF studies were undertaken in a quiet temperature-controlled vascular laboratory (23°C to 25°C) after at least 30 min of rest. A 27-gauge needle was inserted into the brachial artery under local anaesthesia using less than 0.2 ml 1% lignocaine, and saline vehicle or drugs infused at 1ml/min by constant rate infusion pump. FBF was measured by venous occlusion plethysmography using electrically calibrated mercury-in-silastic strain gauges (Hokanson et al., 1975). Radial artery diameter was measured using a high-precision ultrasound scanner. Drugs were infused for at least 5 min and blood flow was measured over the final 1min of infusion, with the mean of 5 measurements used for analysis. The protocols described below were used to assess the effects of non-selective NOS inhibition with L-NMMA and nNOS specific inhibition with SMTC on handgrip exercise +/- reflex sympathetic activation induced by LBNP.

3.2.1 LBNP to generate upper limb sympathetic vasoconstriction

LBNP is the application of subatmospheric pressure to the lower half of the body from the waistline to the feet, in order to retain blood in the veins, reducing venous return and therefore cardiac filling pressures. This process, which mimics hypovolaemia, leads to a global increase in peripheral sympathetic output (and therefore a global increase in peripheral resistance vessel tone) as a mechanism to conserve blood volume centrally (Zoller et al., 1972). This increase in resistance vessel tone is measured as a reduction in FBF.

The LBNP chamber can be made of any substance that will withstand the generated pressure difference, and at the top end there must be a mechanism for ensuring a tight seal around the subject's waist. The LBNP chamber we used was constructed from a wooden base and semi-circular wood/fibreglass tube sealed with rubber tubing. The subject wore a neoprene canoe spray deck around their waist (tightened with a strap), and this was fastened with perspex and clamps to the top end of the chamber to secure a tight seal.

Subatmospheric pressure was generated by an electrically powered rotary vacuum pump connected through a sealed hole at the foot of the chamber, and the chamber pressure was measured using a manometer connected to the inside of the chamber via oxygen tubing. The magnitude of the vacuum stimulus was tightly controlled at -20mmHg using a rheostat connected to the vacuum pump. This was essential, since LBNP greater than -20mmHg leads to reduction in systemic arterial pressure in addition to decreased cardiac filling pressure, suggesting that higher levels of LBNP

affect both arterial baroreceptors and cardiopulmonary receptors (Johnson et al., 1974; Zoller et al., 1972). In contrast, when LBNP is -20mmHg or less, cardiac filling pressure decreases and forearm vascular resistance increases without any changes in arterial parameters including mean pressure or heart rate. This suggests that mild LBNP decreases the stimulus to low-pressure receptors in the cardiopulmonary region without significantly affecting arterial baroreceptor activity (Johnson et al., 1974; Zoller et al., 1972) and that low-pressure cardiopulmonary baroreceptors exert an important influence on forearm vascular resistance during situations of reduced cardiac filling. Meanwhile, LBNP less than -20mmHg increases sympathetic output in muscle branches of the median nerve, again in the absence of any change in arterial parameters (Sundlof and Wallin, 1978).

Taken together, these studies suggest that low-pressure baroreceptors exert an important influence on forearm vascular resistance in response to the decreased cardiac filling pressures induced by mild LBNP, and it is this response that is thought to be regulated by nNOS by Sander and colleagues (Sander et al., 2000).

3.2.2 Protocols

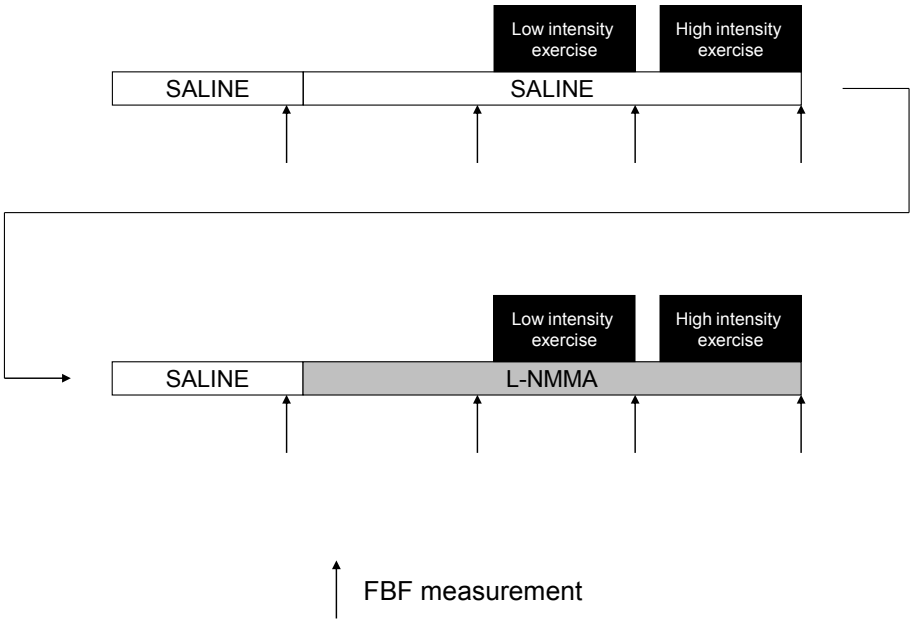
3.2.2.1 Effects of L-NMMA on FBF during handgrip exercise

After brachial artery cannulation, saline was infused and baseline FBF was established after 7 min of infusion. Subjects then performed handgrip exercise using a modified Grip dynamometer (U.S. Gauge, USA) for 3 min at 30pulls/min at low intensity (30% maximal voluntary contraction, MVC) and for 3 min during high intensity (80%

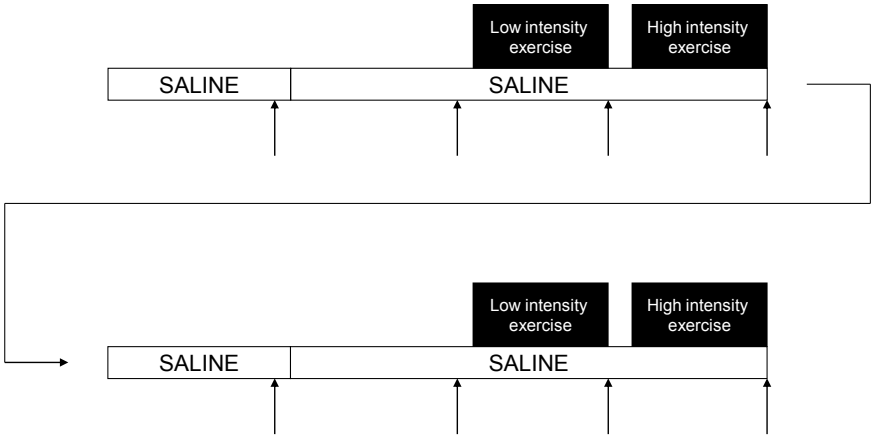
MVC) handgrip exercise (Longhurst et al., 1974). MVC is established by asking the subject to squeeze the dynanometer as tightly as possible. The measurement of FBF by venous occlusion plethysmography requires the arms to be motionless, and therefore FBF measurements were made for 1 min immediately after the cessation of handgrip exercise with the mean of 5 measurements used for analysis. This was done after low intensity handgrip exercise, immediately after which high intensity handgrip exercise was commenced. FBF was again measured as soon as exercise ceased. After a ~25 min recovery period, L-NMMA (2 μmol/min, n=11) was infused for 7 min at rest and during a repeat of the protocol, i.e. further 3 min periods of low and high intensity handgrip exercise. To control for a carry-over effect of the first period of exercise, this protocol was repeated in a separate group of subjects (n=11) with the infusion of saline control in place of L-NMMA during the second period of exercise (Figure 3.1).

Figure 3.1: Schematic of study protocol. FBF was measured using venous occlusion plethysmography. The effect of low and high intensity hand-grip exercise on FBF was measured in the presence of (A) L-NMMA (2 μmol/min) and vehicle and also with (B) vehicle throughout.

A



B


Low intensity exercise
High intensity exercise
SALINE
SALINE
Low intensity exercise
High intensity exercise
SALINE
SALINE

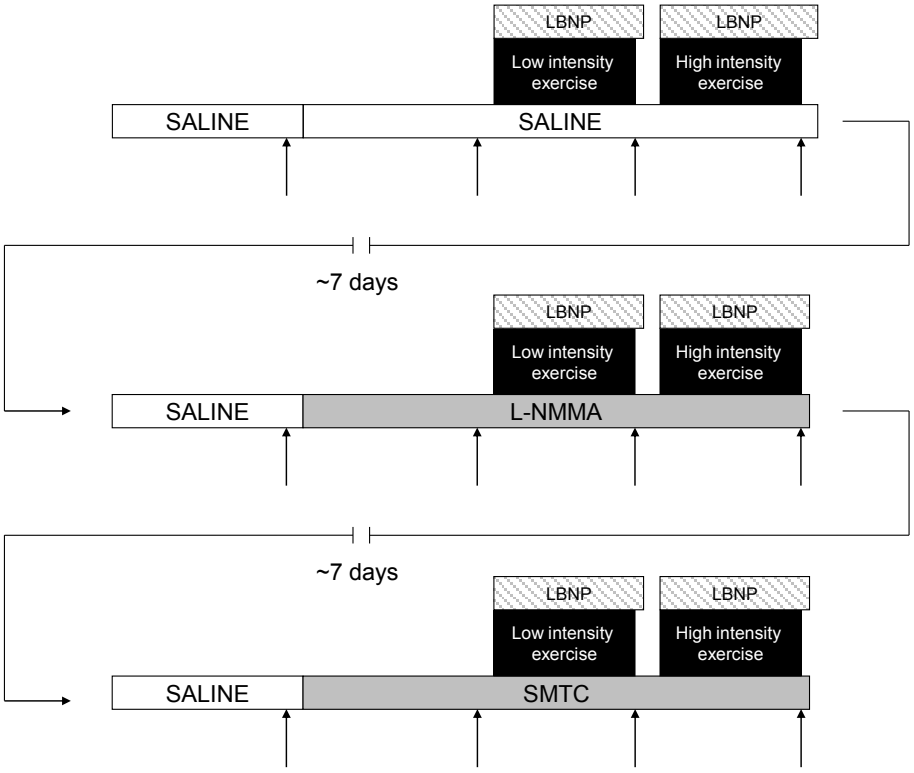
3.2.2.2 Effects of SMTC and L-NMMA on FBF during exercise and LBNP

FBF remained persistently elevated after exercise despite a prolonged period of recovery. This protocol was, therefore, performed on separate days. In summary, on each day, after baseline FBF was established subjects had an infusion of either saline (n=10), SMTC (0.2 μmol/min, n=10) or L-NMMA (2 μmol/min, n=10) at rest and during low and high intensity handgrip exercise in the presence of simultaneous – 20 mmHg LBNP.

A neoprene canoe spray deck was pulled up to the subject's waist before the subject was placed in a LBNP chamber set up on a bed. A tight seal at the top end of the chamber was secured using clamps and the subject lay quietly for 30 min before infusions or measurements were made.

After brachial artery cannulation, saline was infused and baseline FBF established after 7 min infusion. The infusion was then changed to either L-NMMA (2 μmol/min) or SMTC (0.2 μmol/min), which was infused continuously until the end of the study. The same protocol was repeated with saline vehicle throughout as a control, with FBF measurements repeated in the absence of a vasoactive substance. Again, FBF was recorded after a further 7 min to give basal FBF during the infusion of vasoactive substance. MVC was now established and low and high intensity exercise commenced as previously described. LBNP was applied at -20mmHg shortly after the start of exercise and continued beyond cessation of exercise until FBF measurements were completed (Figure 3.2).

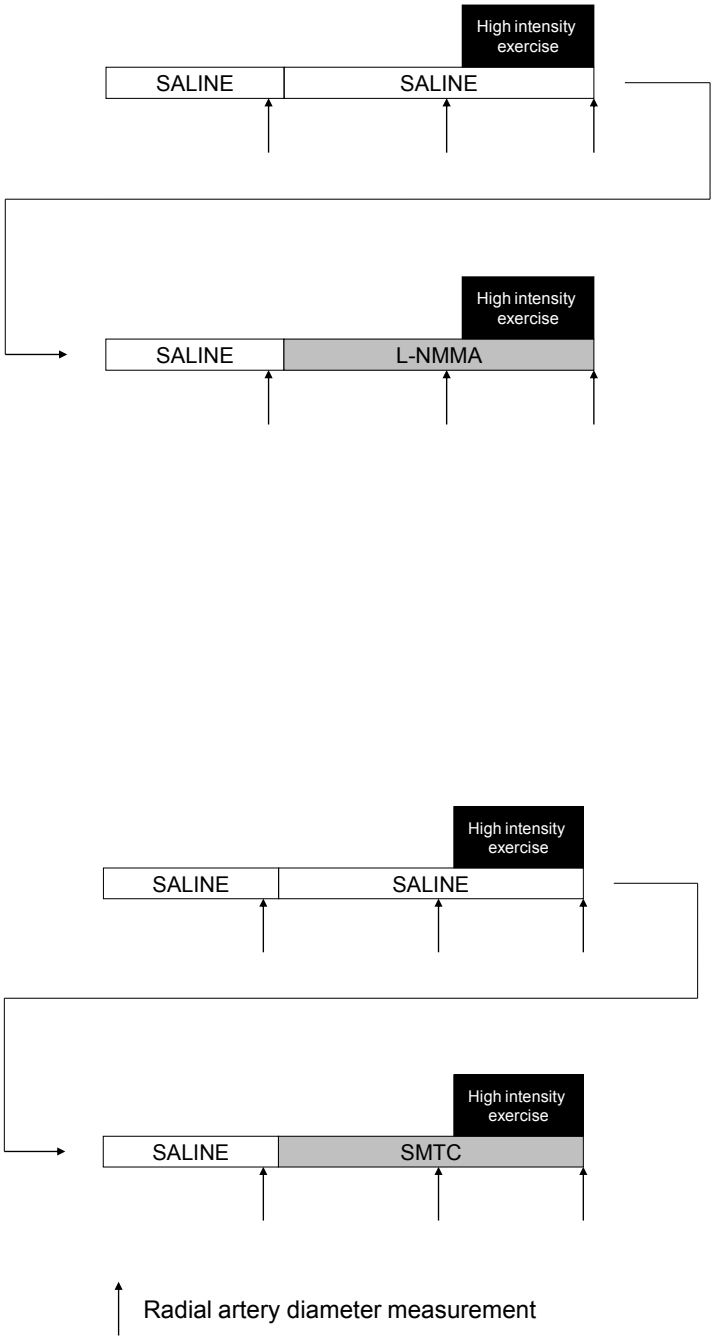
Figure 3.2: Schematic of study protocol. FBF was measured using venous occlusion plethysmography. The effect of simultaneous LBNP with low and high intensity exercise on FBF was measured in the presence of L-NMMA, SMTC and saline control in a cross-over study.



3.2.2.3 Effects of SMTC and L-NMMA on radial artery diameter during exercise

To assess the role of NO in conduit artery vasodilatation rather than flow, radial artery diameter was measured in the presence of L-NMMA or SMTC at rest and during exercise. Subjects attended twice, once receiving either L-NMMA or SMTC, and on the other occasion saline vehicle throughout as control (Figure 3.3). After brachial artery cannulation, saline was infused and baseline radial artery diameter was established after 7 min infusion. After baseline diameter was established, subjects had an infusion of either saline, SMTC (0.2 μmol/min, n=8) or L-NMMA (2 μmol/min, n=8) at rest and during high intensity handgrip exercise. Radial artery diameter measurements were made for 1 min immediately after the cessation of handgrip exercise.

Figure 3.3: Schematic of study protocol. Radial artery diameter was measured using an ultrasound scanner. The effect of high intensity exercise on radial artery exercise was measured in the presence of L-NMMA, SMTC and saline control.



3.2.3 Statistical analysis

Data were summarized as mean ± standard error of the mean (s.e.m.). Vasoconstrictor responses to SMTC and L-NMMA were expressed as the absolute decrease in FBF and percent reduction in FBF from baseline. Vasodilator responses to exercise were expressed as the absolute FBF. This method of expressing FBF responses has previously been shown to be the most reproducible (Walker et al., 2001). Effects of the NOS inhibitors on the blood flow diameter responses were analysed by One-way analysis of variance (ANOVA) or by ANOVA for repeated measures as appropriate. All tests were two-tailed and differences were considered significant when $P<0.05$.

3.3 RESULTS

3.3.1 Pilot study: SMTC dose-response study

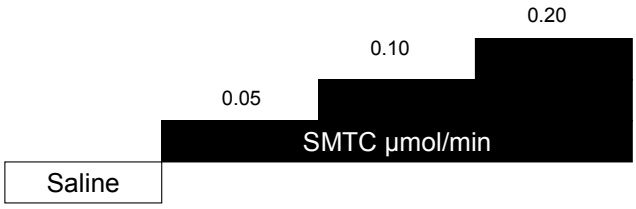
Three initial doses were chosen to be used in these preliminary studies in 3 subjects aged 36.3±3.9 years (Table 3.1), in order to allow construction of a dose-response curve and confirm our labs previous findings of the effect of SMTC on basal FBF (Seddon et al., 2009; Seddon et al., 2008). The three doses of SMTC were 0.05; 0.10 and 0.20 μmol/min, to achieve estimated local concentrations of 2.5; 5 and 10 μmol/L, with each dose being infused for 9 minutes.

Table 3.1: Baseline characteristics of the subjects in the SMTC dose-response study.

Healthy male volunteers (n=3)	
Age (years)	36.3±3.9
Systolic blood pressure [SBP], (mmHg)	122±7.0
Diastolic blood pressure [DBP], (mmHg)	70±2.2
Heart rate (beats per minute)	71± 9.4

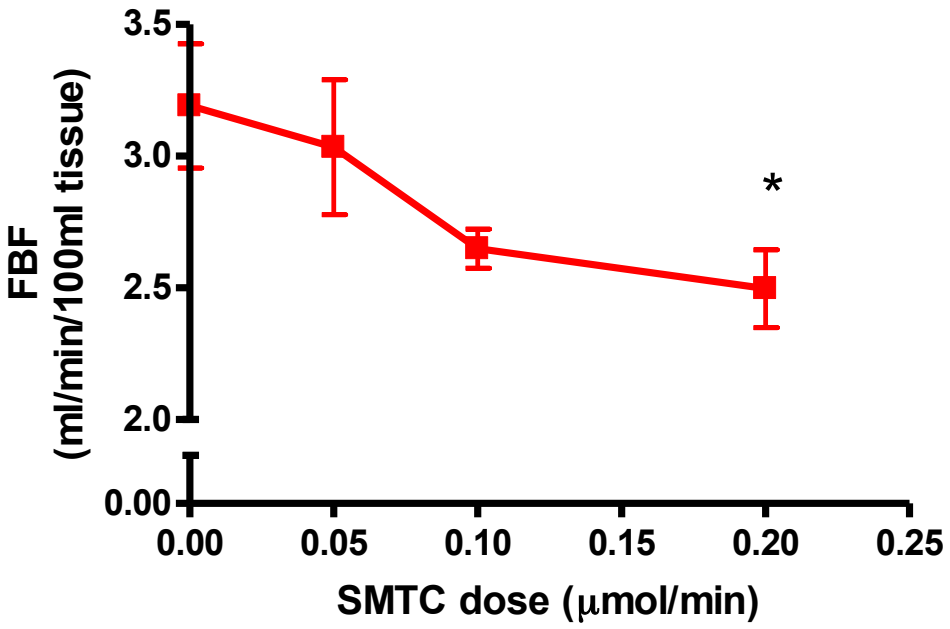
After cannulating the brachial artery, saline was infused for 9 min, with FBF recorded between 7 and 9 min as a measure of baseline FBF. Next, SMTC was infused for 9 min, at 0.05 μmol/min. Again, FBF was recorded between 7 and 9min. This was repeated with two further cumulative doses of SMTC, 0.1 μmol/min and 0.2 μmol/min, infused sequentially for 9 min each (Figure 3.4). Blood pressure indices and heart rate did not change during the infusion of SMTC.

Figure 3.4: Schematic of study protocol. Cumulative doses of SMTC were infused and FBF was measured after each dose, using venous occlusion plethysmography.



SMTC reduced basal blood flow in the infused arm in a dose-dependent manner (Figure 3.5). During saline infusion, baseline FBF was 3.19±0.24 ml/min/100mls tissue. FBF was reduced to 3.03±0.26, 2.65±0.07 and 2.50±0.15 ml/min/100mls tissue with SMTC 0.05, 0.10 and 0.20 µmol/min respectively. Vasoconstrictor effects on FBF are generally expressed as percentage reductions in FBF, therefore SMTC 0.05, 0.1 and 0.2 µmol/min induced reductions of 4.96±3.2, 16.1±6.3 and 21.4±3.5 % respectively ($P<0.05$ only for SMTC 0.2 µmol/min vs. baseline).

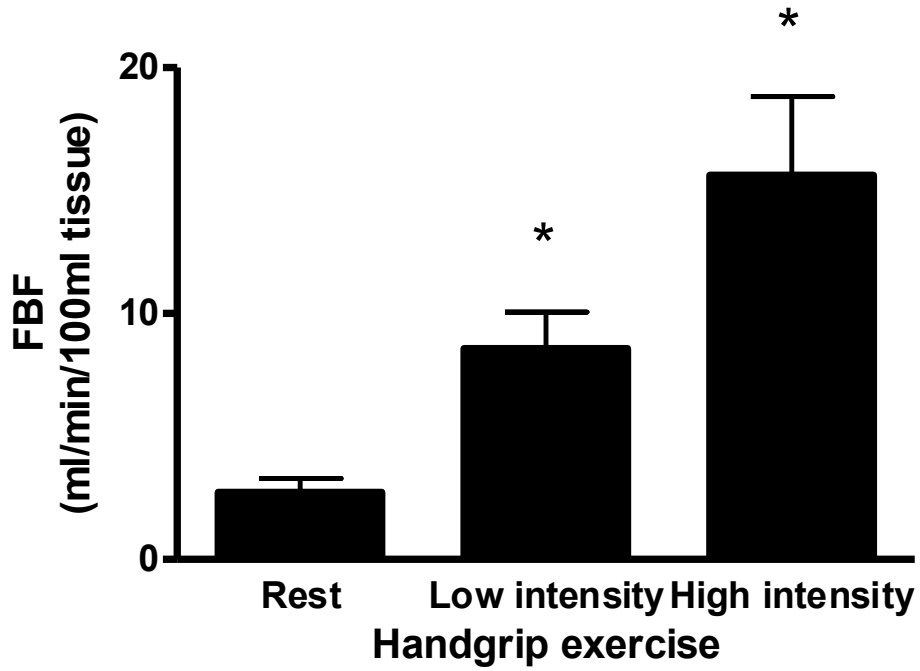
Figure 3.5: Effect of local intra-arterial infusion of SMTC on basal FBF (n=3; *$P<0.05$ SMTC 0.2µmol/min vs baseline)



3.3.2 Pilot study: The effect of handgrip exercise on FBF

Pilot studies were performed to investigate the effect of handgrip exercise on FBF and the length of time required for FBF to return to baseline after handgrip exercise. This was to aid the design of subsequent protocols for studies with NOS inhibitors. FBF was measured by venous occlusion plethysmography as described above. Subjects were asked to perform rhythmic handgrip exercise at low and high intensity as described earlier. Of note, subjects found it simple to complete the period of low intensity exercise. High intensity exercise was significantly more difficult and subjects found that they had exercised almost to exhaustion at the completion of 3 min. 5 subjects aged 32.4±4.7 years were studied. FBF increased from a baseline of 2.72±0.58 to 8.58±1.47 ml/min/100ml during low intensity handgrip exercise ($P<0.05$) and 15.62±3.21 ml/min/100ml during high intensity handgrip exercise ($P<0.05$ vs. baseline and vs. low intensity handgrip exercise) (Figure 3.6).

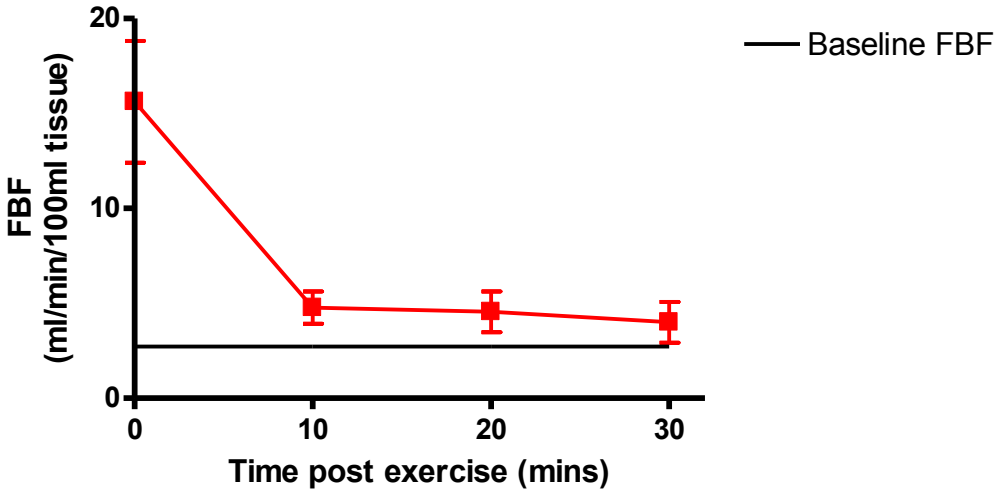
Figure 3.6: FBF at rest (baseline) and during low and high intensity handgrip exercise (n=5, *P<0.05 for low intensity exercise vs. rest, high intensity exercise vs. rest, and low intensity exercise vs. high intensity exercise)



FBF was then measured at different intervals in recovery, up to 30 min after the period of high intensity exercise was completed. This was done to establish the length of time required for FBF to return to baseline. We had to consider the total length of the study as subjects will have an arterial needle in situ, and we intended to minimise the risk of causing any extra discomfort to the subject. We felt that given the length of the exercise protocol, the longest we could allow for a break before repeating the protocol could be 25-30 min. This was from years of experience of arterial cannulation at the Department of Clinical Pharmacology (St. Thomas' Hospital).

The FBF decreased from 15.62±3.21 ml/min/100ml during high intensity exercise (i.e. 0 min into recovery) to 4.78±0.85 after 10 min (P<0.05 vs. baseline), 4.55±1.07 (P=NS vs. baseline) after 20 min, and 4.00±1.07 ml/min/100ml (P=0.11 vs. baseline) 30 min into recovery. At 30 min after exercise, the FBF did not appear to return to baseline (Figure 3.7).

Figure 3.7: FBF after exercise. FBF appears to plateau without quite returning to pre-exercise baseline FBF (depicted by the solid black line).



3.3.3 Effects of L-NMMA on FBF during exercise

Baseline characteristics for the eleven subjects receiving L-NMMA and the eleven control subjects are shown below (Table 3.2). Blood pressure indices and heart rate did not change during the infusion of L-NMMA.

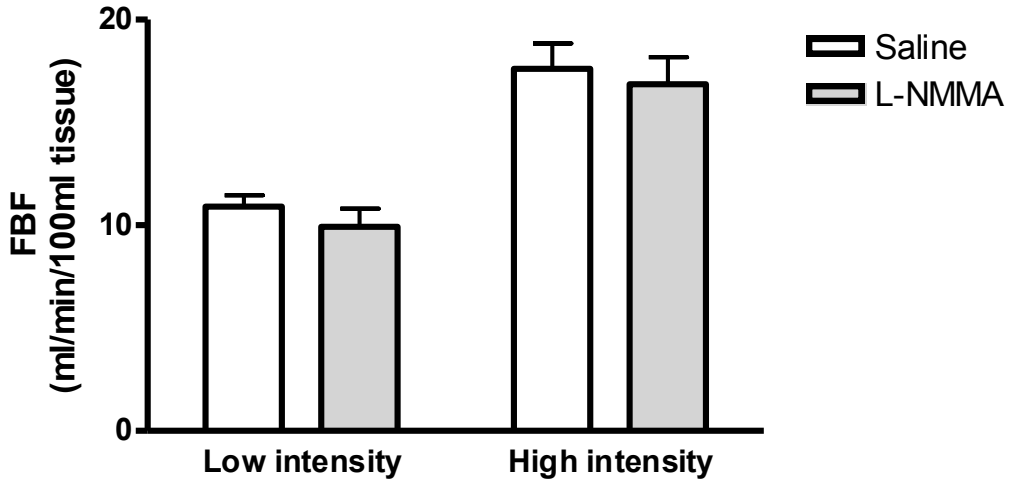
Table 3.2: Baseline characteristics of study subjects in the L-NMMA group and the control group (n=11 both groups).

Healthy male volunteers	Control	L-NMMA
Age (years)	25.9 (± 2.1)	23.9 (± 2.0)
Systolic blood pressure [SBP], (mmHg)	127 (±2.5)	131 (±2.7)
Diastolic blood pressure [DBP], (mmHg)	66 (±1.3)	70 (±1.6)
Heart rate (beats per minute)	68 (±3.7)	64 (± 2.7)

During saline infusion, FBF increased from a baseline of 2.86±0.18 to 10.90±0.56 and 17.61±1.23 ml/min/100ml immediately after low and high intensity handgrip exercise, respectively. A small increase in FBF persisted after exercise despite a 25 min period of recovery (2.86±0.18 ml/min/100ml before exercise vs. 6.20±0.45 ml/min/100ml after 25 min recovery). Nevertheless, the FBF elicited by a second period of low (9.93±0.87, P=NS vs. saline) and high (16.87±1.31, P=NS vs. saline) intensity

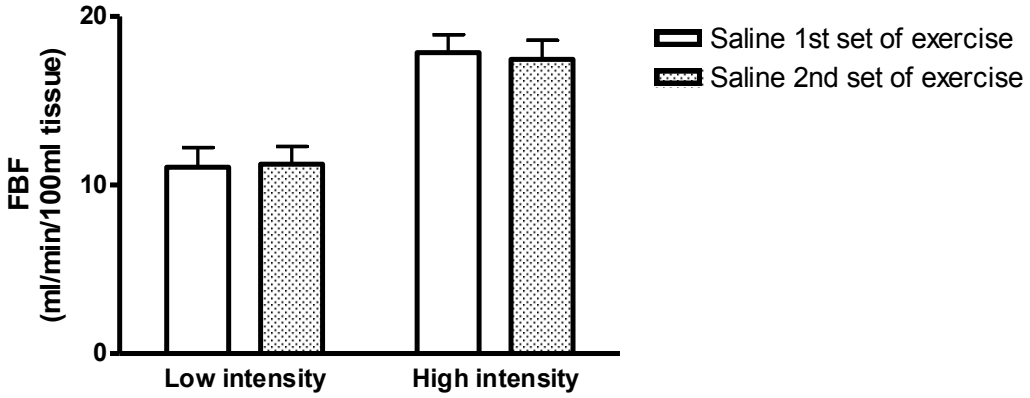
handgrip exercise in the presence of L-NMMA was similar to that observed during saline infusion (Figure 3.8).

Figure 3.8: A comparison of FBF whilst undertaking low and high intensity exercise, during saline or L-NMMA infusion. There was no significant difference in FBF during L-NMMA infusion when compared to saline during either low intensity exercise (n=11, P=NS) or high intensity exercise (n=11, P=NS).



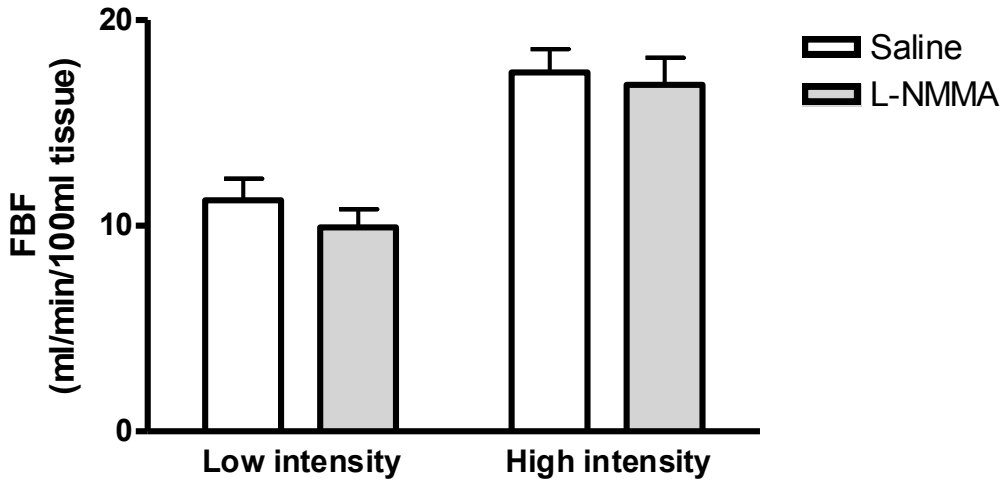
Due to the small but persistent increase in FBF after the first set of exercise, the study was repeated with saline vehicle throughout. Again, a small increase in FBF persisted despite a 25 min period of recovery (2.70±0.29 ml/min/100ml before exercise vs. 4.97±0.51 ml/min/100ml after 25 min recovery). However, the FBF elicited was similar during the first and second exercise periods of low (11.06±1.16 vs. 11.24±1.04 ml/min/100ml respectively, P=NS) and high intensity (17.86±1.06 vs. 17.46±1.14 ml/min/100ml respectively, P=NS) (Figure 3.8).

Figure 3.9: A comparison of FBF whilst undertaking low and high intensity handgrip exercise during saline infusion. The second set of exercise was carried out after a 25min break/recovery from the first set of exercise and hence FBF had not quite returned to baseline. However, there was no significant difference in FBF when compared to the first set of handgrip exercise during either low intensity (n=11, P=NS) or high intensity (n=11, P=NS).



When comparing the second set of exercises for each study (i.e. second set of exercise in the presence of L-NMMA vs. second set of exercise in the control study in the presence of saline vehicle) there was no difference in the FBF elicited during low intensity (9.93±0.87 vs. 11.24±1.04 ml/min/100ml respectively, P=NS) or high intensity (16.87±1.31 vs. 17.46±1.14 ml/min/100ml respectively, P=NS) handgrip exercise (Figure 3.10).

Figure 3.10: A comparison of FBF whilst undertaking low and high intensity exercise, during saline or L-NMMA infusion, carried out in separate studys. There was no significant difference in FBF during L-NMMA infusion when compared to saline during either low intensity exercise (n=11, P=NS) or high intensity exercise (n=11, P=NS).



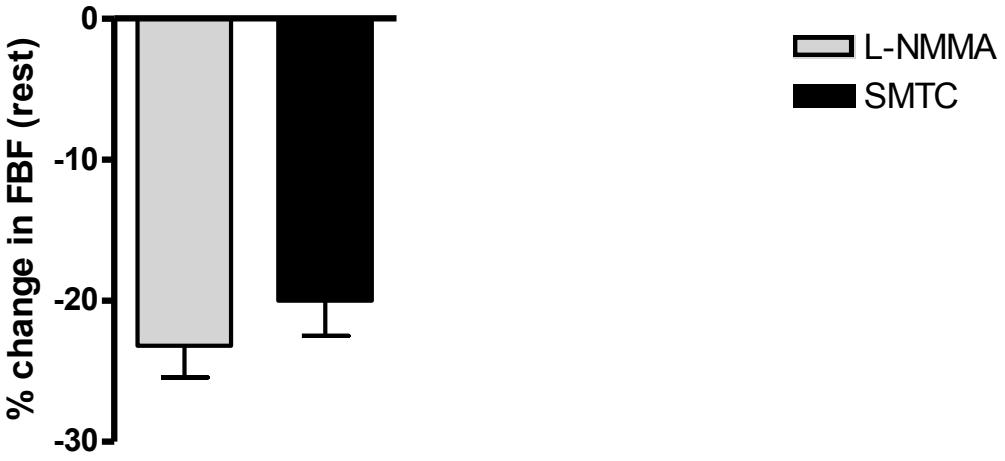
3.3.4 Effect of L-NMMA and SMTC on FBF during exercise and LBNP

15 healthy male volunteers aged 25.9±2.1 years were studied (Table 3.3). On separate study days, L-NMMA (2 μmol/min) and SMTC (0.2 μmol/min) reduced resting blood flow to a similar degree, by 23.2±2.2 % (from 5.40±0.72 to 4.21± 0.61 ml/min/100ml, P<0.001) and 19.6±2.5 % (from 5.46±0.73 to 4.42±0.60 ml/min/100ml, P<0.001), respectively (L-NMMA vs. SMTC P=NS) (Figure 3.11).

Table 3.3: Baseline characteristics of the study subjects.

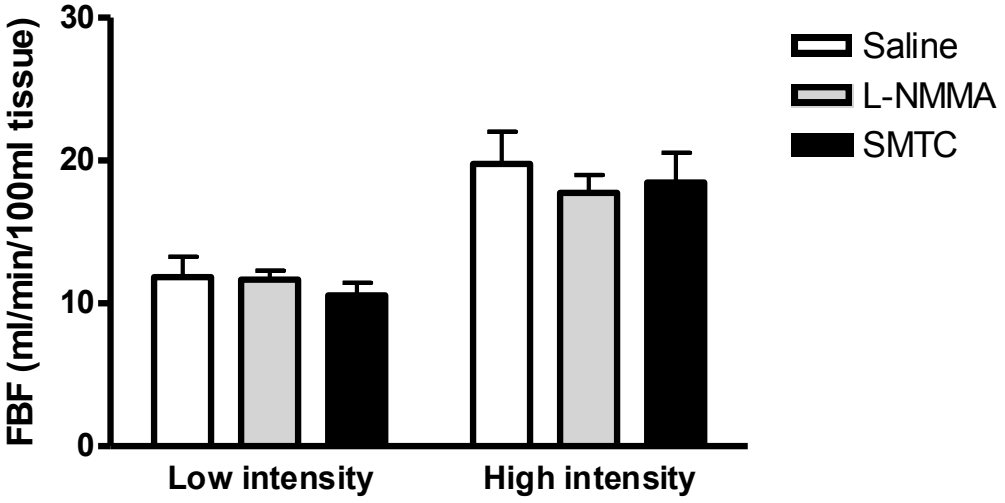
Healthy male volunteers	
Age (years)	25.9±2.0
Systolic blood pressure [SBP], (mmHg)	126±1.7
Diastolic blood pressure [DBP], (mmHg)	68±2.0
Heart rate (beats per minute)	64± 2.2

Figure 3.11: Basal reduction in FBF with L-NMMA and SMTC (n=10, P=0.29).



During handgrip exercise and simultaneous LBNP, there was no significant effect of either L-NMMA or SMTC on FBF induced by exercise at low intensity (11.66±0.62 vs. 10.57±0.85 vs. 11.84±1.43 ml/min/100ml tissue change during L-NMMA, SMTC and saline vehicle respectively; n=10, L-NMMA vs. SMTC P=NS, L-NMMA vs. saline P=NS, SMTC vs. saline P=NS) or high intensity (17.74±1.25 vs. 18.47±2.06 vs. 19.76±2.25 ml/min/100ml tissue change during L-NMMA, SMTC and saline vehicle respectively; n=10, L-NMMA vs. SMTC P=NS, L-NMMA vs. saline P=NS, SMTC vs. saline P=NS) (Figure 3.12). Results did not differ when data were analysed using a one-way ANOVA on all subjects or a repeated-measurements ANOVA restricted to subjects that attended all study days and thus had data during infusion of saline, LNNMA or SMTC.

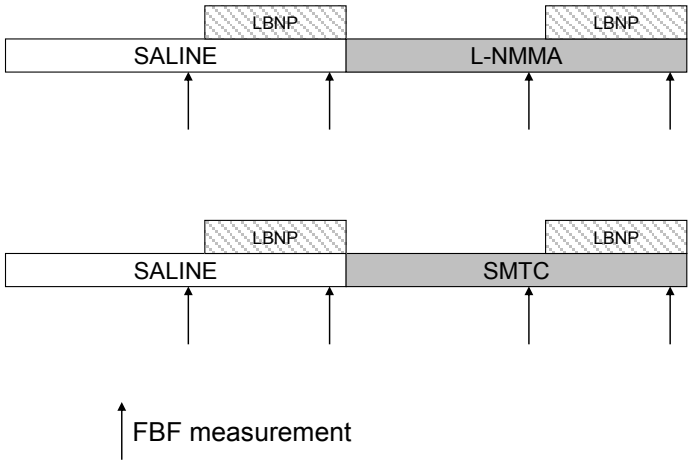
Figure 3.12: A comparison of the FBF during low and high intensity exercise and LBNP whilst infusing L-NMMA or SMTC. There is no significant difference in FBF during L-NMMA or SMTC infusion when compared to saline, for either low intensity (n=10, P=NS for both) or high intensity exercise (n=10, P=NS for both).



3.3.5 Effects of SMTC and L-NMMA on FBF during LBNP alone

This particular study was carried out in our department by Seddon and colleagues but the data has been further analysed in this thesis as it is relevant to the work in this chapter. Saline vehicle was infused for 15 min to establish baseline FBF, followed by infusion of SMTC (0.2 μmol/min for 15 min, n=8) and, in a separate group of subjects, L-NMMA (2 μmol/min for 15 min, n=8). During the final 3 min of each infusion period, reflex sympathetic activation was induced by applying -20 mmHg LBNP (Figure 3.13).

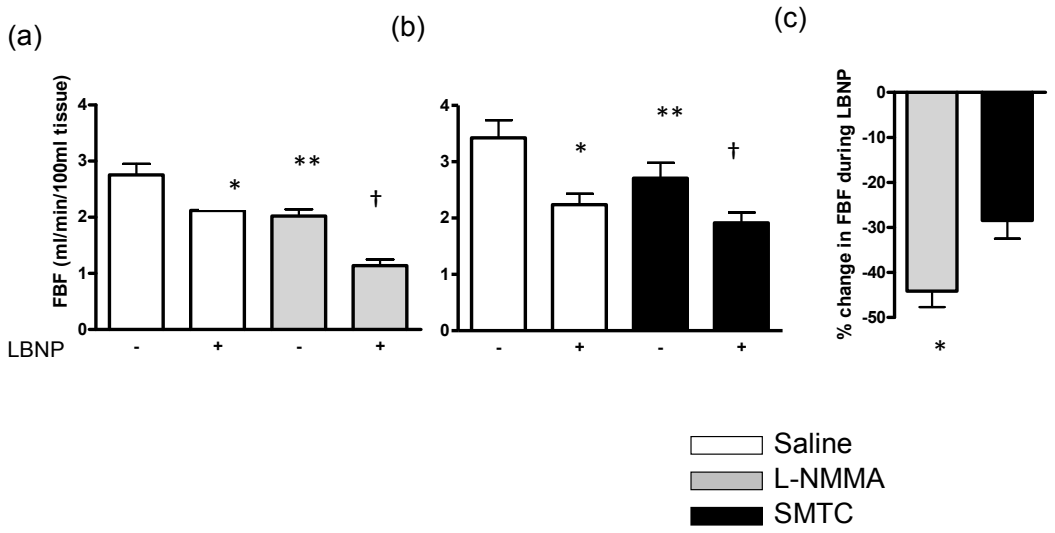
Figure 3.13: Schematic of study protocol. The effect of LBNP on FBF was measured during infusion of L-NMMA (2 μmol/min) and vehicle, and SMTC (0.2 μmol/min) and vehicle.



At rest and in the absence of LBNP, both L-NMMA (2 μmol/min) and SMTC (0.2 μmol/min) significantly reduced FBF by 25.8±3.0 % (from 2.76±0.20 to 2.02±0.12 ml/min/100ml, P<0.001, figure 3.14a) and 20.6±3.3 % (from 3.42±0.31 to 2.71±0.27 ml min/100ml, P<0.01, P=NS compared to L-NMMA, figure 3.13b), respectively. LBNP (-20mmHg) reduced FBF by 23.4±5.7 % (from 2.75±0.20 to 2.12±0.22 ml/min/100ml, P<0.01) during saline infusion and by 44.2±3.5 % in the presence of L-NMMA (from 2.02±0.12 to 1.14±0.11 ml/min/100ml, P<0.01 vs. saline, figure 3.13a). By contrast, the reduction in FBF by LBNP during infusion of SMTC did not differ significantly from that observed during saline infusion (from 3.42±0.31 to 2.24±0.20 ml/min/100ml, i.e., a 34.1±3.0 % reduction, P<0.001 during saline and from 2.71±0.27 to 1.92±0.18 ml/min/100ml, i.e., a 28.5±4.0 % reduction during SMTC, P=NS

between interventions, figure 3.14b). Thus, the relative reduction in FBF in response to LBNP was significantly greater in the presence of L-NMMA than in the presence of SMTC (44.2±3.5 vs. 28.5±4.0 %, P<0.01, figure 3.14c).

Figure 3.14: (a) FBF at rest and during LBNP during infusion of saline and L-NMMA. * P<0.01 vs. saline with no LBNP; ** P<0.001 vs. saline with no LBNP; † P<0.001 vs. L-NMMA with no LBNP. (b) FBF at rest and during LBNP during infusion of saline and SMTC. * P<0.001 vs. saline with no LBNP; ** P<0.01 vs. saline with no LBNP; † P<0.01 vs. SMTC with no LBNP. (c) The % change in FBF during L-NMMA and SMTC with LBNP. * P<0.01 vs SMTC. (+ LBNP; - no LBNP).



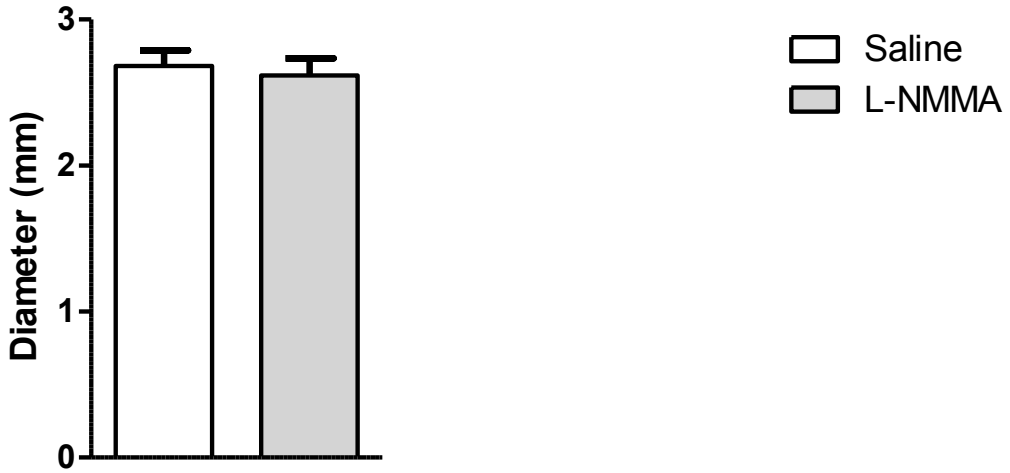
3.3.6 Effects of SMTC and L-NMMA on radial artery diameter during exercise

17 healthy male volunteers (3 volunteers took part in both the L-NMMA and the SMTC study) were studied. Only high intensity exercise was performed, to ensure a large enough vasodilatation response to be detectable. At rest, radial artery diameter did not change in the presence of L-NMMA (from 2.49±0.15 before to 2.48±0.13mm after L-NMMA; P=NS) nor SMTC (from 2.37±0.07 before to 2.36±0.08mm after SMTC; P=NS).

During handgrip exercise, radial artery diameter increased in the saline control study in both group of subjects. In the L-NMMA group, radial artery diameter increased from 2.50±0.13 to 2.68±0.11 mm (P<0.01), and in the SMTC group radial artery diameter increased from 2.49±0.14 to 2.79±0.15 mm (P<0.05). Radial artery diameter also increased during exercise in the presence of L-NMMA (from 2.48±0.13 to 2.62±0.12mm, P<0.01) and SMTC (from 2.36±0.08 to 2.50±0.07 mm, P<0.001), and this was not significantly different to the increase seen during saline control (P=NS, L-NMMA vs saline; P=NS, SMTC vs saline) (Figure 3.14).

Figure 3.15: A comparison of the radial artery diameter during high intensity exercise whilst infusing L-NMMA or SMTC. There is no significant difference in radial artery diameter during L-NMMA (n=8, P=NS) or SMTC (n=8, P=NS) infusion when compared to saline.

A



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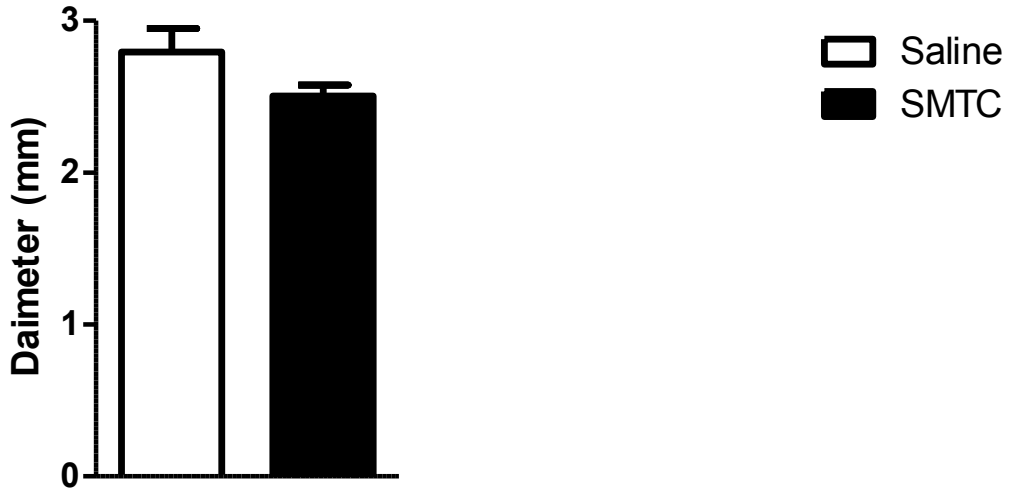


3.4 DISCUSSION

Until recently, it had been assumed that the NO that regulates local blood flow under physiological conditions in humans derives exclusively from eNOS. However, first-in-human studies with the nNOS-selective inhibitor SMTC showed that the basal regulation of vascular tone in both the forearm and coronary circulations is mediated by nNOS whereas eNOS mediates relaxant responses to pharmacological and shear stress stimuli (Seddon et al., 2009; Seddon et al., 2008). Cutaneous vasodilatation induced by heat stress is also mediated by nNOS in humans (Kellogg et al., 2008b). The autonomic nervous system could activate local release of NO from nNOS through a neuronal link and/or eNOS through local release of neurotransmitters such as norepinephrine. To our knowledge, this is the first study to examine the relative role of local nNOS- and eNOS-mediated vasomotor responses to physiological stimuli that modulate activity of the sympathetic and possibly other neural pathways within the autonomic nervous system. In the study carried out by colleagues in our department we found that non-isoform selective NOS inhibition with L-NMMA augmented the forearm vasoconstrictor response to LBNP whereas selective nNOS inhibition (despite causing a reduction in basal forearm blood flow similar to that observed with L-NMMA) had no significant effect on the vasoconstrictor response to LBNP. This suggests that reflex sympathetic vasoconstriction is partially opposed by local eNOS- but not by nNOS-derived NO in the resting muscle. These results are consistent with observations in the rat hind limb where non-selective NOS inhibition with N-nitro-L-arginine methyl ester (L-NAME) increases muscle vasoconstriction in response to lumbar sympathetic nerve stimulation (Thomas and Victor, 1998). The simplest explanation for the augmented vasoconstriction to sympathetic stimulation after NOS

inhibition is that norepinephrine released from sympathetic nerves in response to LBNP stimulates eNOS through alpha or beta-adrenergic receptors, actions that have previously been described in animals and humans (Kneale et al., 2000).

Increases in blood flow during exercise depend upon short-term adjustments and interplay between autonomic influences and local regulatory factors. These results enhance our understanding of the role of NO-mediated circulatory control by demonstrating that neither nNOS nor eNOS appear to subserve an obligatory role in regulating blood flow during exercise or in modulating the sympathetic vasoconstrictor response in exercising muscle in humans *in vivo*.

Previous human and animal studies examining the role of NO during functional hyperaemia have yielded conflicting results. Copp and colleagues found that nNOS inhibition with SMTC reduced baseline hindlimb muscle blood flow in the rat, but not total hindlimb muscle blood flow during exercise (Copp et al., 2010). However, many animal models do suggest a potential role for NO, and more specifically nNOS-derived NO in regulating blood flow and oxygenation in skeletal muscle by blunting the vasoconstrictor response to α-adrenergic activation during dynamic exercise (functional sympatholysis) and/or contributing to exercise-induced vasodilatation. These include a dog hind limb model of exercise (Buckwalter et al., 2004) and nNOS deficient mice (Fadel et al., 2003; Grange et al., 2001; Kobayashi et al., 2008; Lai et al., 2009; Percival et al., 2008; Percival et al., 2010; Thomas et al., 1998). In the *mdx* mouse, a mouse model of DMD where dystrophin deficiency results in reduced nNOS expression in skeletal muscle, the normal ability of muscle contraction to attenuate α-adrenergic vasoconstriction is defective (Thomas et al., 1998). Kobayashi and

colleagues have suggested that nNOS in skeletal muscle contributes to increased blood flow after mild exercise in mouse models (Kobayashi et al., 2008).

In humans, a number of studies have shown a reduction in FBF during exercise in response to non-selective NOS inhibition with L-NMMA or L-NAME (Endo et al., 1994; Gilligan et al., 1994; Gordon et al., 2002; Wray et al., 2011). However, the size of the effects has been small and, when compared to a vasoconstrictor control with similar effects on resting blood flow, L-NMMA has been found to have no significant effect on blood flow responses during exercise (Dinenno and Joyner, 2003). Chavoshan and colleagues examined the effect of a reflex increase in sympathetic efferent activity (induced by LBNP) on muscle perfusion (assessed from measurement of muscle oxygenation by NIRS) during exercise (Chavoshan et al., 2002). Systemic NOS inhibition with L-NAME completely reversed the blunted vasoconstrictor response to LBNP in the exercising forearm. However, such results could be influenced by the reflex response to the systemic effects of L-NAME (which include a rise in MAP). Dinenno and Joyner examined the effects of local NOS inhibition with L-NMMA or L-NAME on FBF responses during handgrip exercise and whilst stimulating release of endogenous norepinephrine (by intra-arterial infusion of tyramine). Neither NOS inhibitor restored the vasoconstrictor response to local tyramine-stimulated norepinephrine release during exercise, arguing against a role for NO in functional sympatholysis (Dinenno and Joyner, 2003). As described earlier, Sander and colleagues found that both the LBNP-induced decrease in resting forearm vascular conductance and the decrease in tissue oxygenation during hand grip exercise were attenuated in controls, but not in children with DMD. The authors attributed these effects to the loss of skeletal muscle nNOS-derived NO in children with DMD

(Sander et al., 2000). However, dystrophic muscle is characterized by changes in the expression and function of a number of proteins (Straub and Campbell, 1997), raising a question as to whether the impaired response to sympathetic stimulation in DMD were specific for nNOS deficiency.

Our results using L-NMMA showed no significant effect of L-NMMA to blunt functional hyperaemia. Similarly, even in the face of increased sympathetic stimulation with LBNP, we found no significant effect of either L-NMMA or SMTC on FBF responses immediately after exercise. These results confirm the findings of Dinenno and Joyner using a different sympathetic stimulus and using both non-selective and selective inhibitors of NOS (Dinenno and Joyner, 2003) measuring blood flow and conduit artery diameter using Doppler. It is likely that there are multiple mechanisms involved in exercise hyperaemia and functional sympatholysis including metabolic mediators such as adenosine, ATP, potassium, hypoxia, and hydrogen ions that may link blood flow to metabolic demands (Clifford and Hellsten, 2004). Noradrenergic, noncholinergic peptides such as calcitonin gene-related protein may also be involved (Hasbak et al., 2002). These results combined with those of other investigators showing the lack of effect of inhibition of NOS on exercise-induced hyperaemia are consistent with the proposal by Clifford and Hellsten that there is a redundancy of vasodilators contributing to exercise-induced hyperaemia where one vasoactive compound may take over when the formation of another is inhibited (Clifford and Hellsten, 2004).

3.4.1 Study limitations

These results pertain to healthy men, and we cannot extrapolate our results to women or subjects with cardiovascular risk factors. Due to the limited sample size we cannot exclude a small effect of eNOS/nNOS on functional sympatholysis. Our study involved acute NOS inhibition and results could differ from those of studies involving chronic nNOS inhibition or absent nNOS such as in a knock-out murine model. SMTC has relatively high specificity for nNOS, and in order to be sure that SMTC was acting specifically on nNOS, we used a concentration previously shown not to inhibit eNOS mediated responses (Seddon et al., 2009; Seddon et al., 2008) but which reduced basal forearm blood flow to a similar degree to concentrations of L-NMMA, that inhibit eNOS mediated responses. However, should more specific inhibitors of nNOS become available for human use, it would be advisable to use these to confirm our findings.

Measurements were made immediately after the cessation of handgrip exercise and we have extrapolated these results to demonstrate the effect during exercise, as many researchers have previously (Endo et al., 1994; Gilligan et al., 1994; Gordon et al., 2002; Wilson and Kapoor, 1993). It is therefore feasible that we are observing the effect on recovery post-exercise rather than exercise itself, and that these may differ. Measurements during exercise were not feasible as the measurement of FBF with venous occlusion plethysmography requires the arms to be motionless, However, the results are similar to some researchers who have used other techniques during the actual exercise period, such as Doppler ultrasound (Dinenno and Joyner, 2003; Green et al., 2005).

3.4.2 Conclusion

In these studies, we used intra-arterial infusions of SMTC and L-NMMA in healthy male volunteers to investigate for the first time its effects on FBF during exercise and reflex sympathetic activation in humans *in vivo*. We found that neither NOS isoform plays an obligatory role in functional sympatholysis during exercise. This suggests that animal models suggesting a role for nNOS in functional sympatholysis may not be relevant to human physiology.

CHAPTER 4:

THE ROLE OF nNOS VERSUS eNOS IN THE REGULATION OF CORONARY VASOMOTOR TONE DURING PACING

4.1 INTRODUCTION

Intricate changes in microvascular tone are responsible for regulation and distribution of myocardial blood flow. NO plays a key role in this complex process regulating vessel tone at rest as well as during episodes of increased flow in response to shear stress or agonist stimulation (Joannides et al., 1995; Moncada and Higgs, 1993; Moncada and Higgs, 2006). Previously thought to derive solely from eNOS in the endothelium of blood vessels, it is now clear that NO synthesised from nNOS plays an equally important role in regulating blood flow. Previous investigations from our group have shown that coronary artery infusion of SMTC, a selective inhibitor of nNOS, reduces human basal CBF *in vivo*, but does not block the vasodilator response to substance P, an eNOS agonist (Seddon et al., 2009). In contrast, substance P-induced increases in coronary blood flow were inhibited by the non-selective NOS inhibitor L-NMMA. This suggests that regulation of basal vascular tone, and hence blood flow at rest, may primarily be dependent upon the action of vascular nNOS as

opposed to eNOS as previously thought. These findings concur with evidence from other animal species where nNOS-derived NO has similarly been shown to have a paracine vasoregulatory influence in different vascular beds (Chi et al., 2003; Ichihara et al., 1998; Thomas et al., 1998; Vallon et al., 2001).

In addition, NO is believed to be one of a number of paracrine mediators that regulate myocardial blood flow in response to increased metabolic demand such as that encountered during exercise (Buckwalter et al., 2004; Endo et al., 1994; Gilligan et al., 1994; Gordon et al., 2002; Grange et al., 2001; Sander et al., 2000; Thomas et al., 1998). However, there is no data on the relative contribution of different NOS isoforms to this process in the human coronary circulation.

4.1.1 Study aims

The aim of the present study was to investigate the relative contribution of eNOS- and nNOS-derived NO in the regulation of epicardial and microvascular tone during pacing-induced changes in flow in a cardiac catheter laboratory setting.

4.2 METHODS

The studies were approved by the local research ethics committee (King's College Hospital), and all participants provided written informed consent. The studies conformed to the standards set by the latest revision of the Declaration of Helsinki.

Patients undergoing diagnostic cardiac catheterization for atypical chest discomfort, who had angiographically smooth unobstructed coronary arteries, were recruited to the study. Subjects with valvular heart disease, left ventricular hypertrophy or systolic dysfunction and significant renal, hepatic or inflammatory disease were excluded. Studies were performed in the morning after an overnight fast. Any vasoactive drugs were discontinued and subjects refrained from alcohol, caffeinated drinks and smoking on the day of the study. Patients were given a formal patient information sheet and all procedures and their associated risks were explained. Subjects were randomly assigned to receive saline vehicle and then either SMTC (0.0625 μmol/min) or L-NMMA (25 μmol/min) during a second incremental pacing protocol. The doses of these agents have been shown to inhibit nNOS and both nNOS and eNOS, respectively (Seddon et al., 2009).

4.2.1 Protocol

The study protocol is summarised in Figure 1. Cardiac catheterization was performed via the right femoral arterial and venous routes in a quiet, temperature-controlled, cardiac catheterization laboratory with digital cineangiography. After completion of diagnostic coronary angiography, a standard temporary pacing wire was positioned in

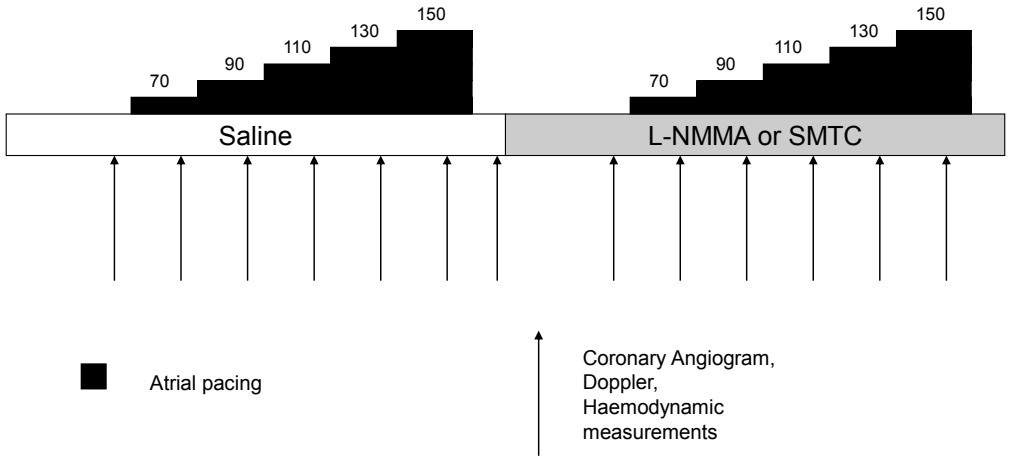
the right atrium and a 0.014-in intra-coronary Doppler wire (FloWire, Volcano Therapeutics Inc, Rancho Cordova, CA, USA) advanced through a 6F guiding catheter into a straight, non-overlapping and side-branch free segment of the proximal left coronary artery. Either the left anterior descending (LAD) or circumflex (LCx) arteries were used as detailed in Table 1. The Doppler wire was interfaced with a real-time spectral analysis system (ComboMap Pressure and Flow system, Volcano Therapeutics Inc, Rancho Cordova, CA, USA) to derive continuous Doppler traces and corresponding average peak velocity (APV) values.

Changes in the diameter of the study artery was calculated using an automated Philips quantitative coronary angiography (QCA) edge detection system in a 2.5-5mm length segment of vessel approximately 2.5 mm distal to the tip of the Doppler wire. Angiographic images were obtained with the study artery positioned at the isocentre without altering the angle of projection throughout the study. CBF was calculated from the product of corresponding APV value and QCA-derived coronary artery diameter (1/2 x APV x coronary cross-sectional area) (Doucette et al., 1992) at pre-specified time points as outlined below and in Figure 4.1. Saline vehicle was infused directly into the coronary artery followed by either SMTC (0.0625 μmol/min) or L-NMMA (25 μmol/min) via the guiding catheter at an infusion rate of 2 ml/min. All results were recorded digitally and analysed offline in a blinded fashion at the end of the study by the same investigator.

Changes in CBF were measured in response to incremental atrial pacing (to increase metabolic demand) before and after intra-coronary infusion of vehicle and SMTC/L-NMMA. APV was initially measured in the presence of saline vehicle at baseline and

after 20 beats per minute (bpm) paced increments in heart rate every 2 min up to a maximum heart rate of 150 bpm or the occurrence of rate-related temporary atrio-ventricular block. APV was then allowed to return to baseline. This was followed by a 7 min intra-coronary infusion of a NOS inhibitor (STMC or L-NMMA). The pacing protocol was then repeated in the presence of the NOS inhibitor. To avoid hyperaemic effects of angiographic contrast influencing CBF calculations at each stage of the protocol, APV recordings were made immediately prior to obtaining the corresponding angiographic image. Furthermore, the protocol progressed to the next stage only after the APV value had returned to its pre-contrast reading. Aortic pressure and ECG were recorded to correspond to every CBF measurement.

Figure 4.1: Schematic diagram of the protocol. Incremental pacing from 70 bpm up to 150 bpm was carried out with measurements of average peak velocity (APV), blood pressure, ECG and coronary angiography after each step. This was in the presence of intra-coronary saline and then either L-NMMA or SMTC.



4.2.2 Statistical analysis

Data was recorded as mean ± SEM. Vasoconstrictor responses to SMTC and L-NMMA were calculated as percentage decrease in basal blood flow. Effects of the NOS inhibitors and pacing on the blood flow responses were analysed by One-way analysis of variance (ANOVA) or by ANOVA for repeated measures as appropriate. All tests were two-tailed and differences were considered significant when $P<0.05$. All graphs demonstrating results with pacing are plotted against both heart rate (HR) and cardiac workload, which was calculated as mean arterial pressure (MAP) x (HR). Coronary vascular resistance (CVR) was calculated as MAP/CBF (Quyyumi et al., 1995a).

4.3 RESULTS

Twenty patients (11 male, mean age 57±3.2 years) were studied, 10 in each group receiving either SMTC or L-NMMA. Baseline characteristics were similar between the SMTC and L-NMMA groups (Table 4.1). None of the subjects developed an adverse reaction to the study drugs, symptoms of ischaemia or changes in the surface 12 lead ECG.

Table 4.1: Baseline characteristics of patients. There was no significant difference in the characteristics between the 2 groups of patients.

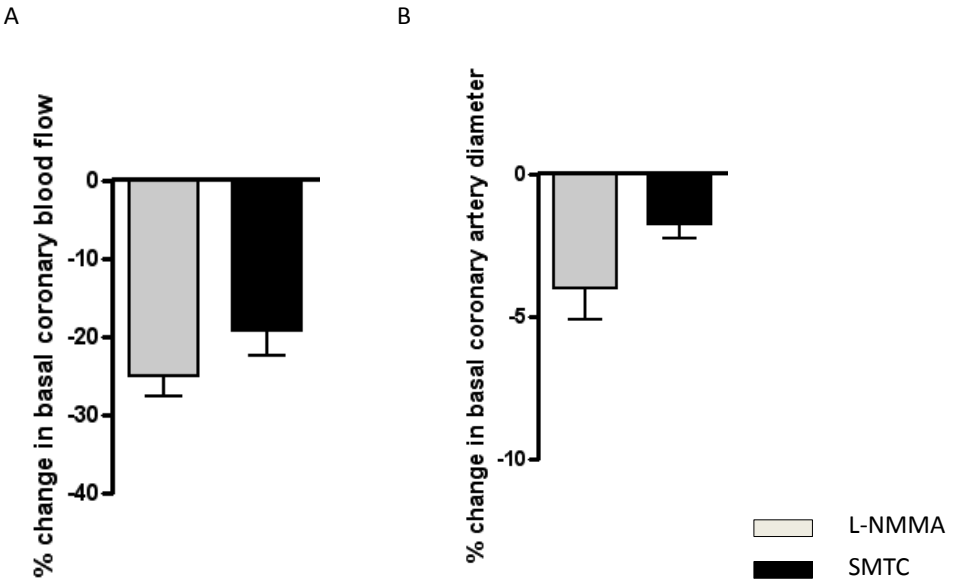
	SMTC [n = 10]	L-NMMA [n = 10]	
Age (years)	58.7±2.9	57.1±3.7	(P=0.73)
Male/ Female, n	6 / 4	5 / 5	
Cardiovascular risk factors:			
Smoker, n	4	4	
Hypertension, n	5	3	
Diabetes mellitis, n	4	2	
Hypercholesterolaemia, n	3	7	
Family history, n	1	1	
Systolic BP (mmHg)	128±7.9	139±7.8	(P=0.30)
Diastolic BP (mmHg)	77±4.1	74±2.3	(P=0.79)
Study artery, n			
LAD	5	6	
LCx	5	4	

4.3.1 Influence of L-NMMA and SMTC on basal CBF

Intra-coronary infusion of L-NMMA or STMC both reduced basal CBF to a similar extent (P=NS) (Figure 4.2A). L-NMMA (25 μmol/min) reduced basal flow by 25.0±2.68 %, from 59.7±9.52 to 43.9±6.47 ml/min (P<0.01) and SMTC (0.625 μmol/min) by 19.2±3.25%, from 70.0±8.16 to 55.8±6.57 ml/min (P<0.001). As with changes in CBF, there was a similar reduction in basal coronary artery diameter in response to L-NMMA and SMTC (P=NS) (Figure 4.2B). L-NMMA reduced coronary arterial diameter from 2.66±0.22 to 2.54±0.20 mm (P<0.01) and SMTC from 2.53±0.12 to 2.48±0.11 mm (P<0.01).

There was no change in blood pressure with either intra-coronary L-NMMA (MAP: 96.7±4.48 to 100.6±5.54 mmHg, P=NS) or SMTC (MAP: 97.8±6.09 to 100.0±7.35 mmHg, P=NS), as previously reported (Seddon et al., 2009).

Figure 4.2: Effect of SMTC and L-NMMA on basal coronary tone. (A) Percentage reduction in basal coronary blood flow after SMTC (n=10) and L-NMMA (n=10). SMTC and L-NMMA both significantly reduced basal coronary artery blood flow to a similar extent (p=NS SMTC vs L-NMMA). (B) Effect of SMTC and L-NMMA on epicardial conduit artery tone. SMTC and L-NMMA both significantly reduced basal coronary artery diameter to a similar extent (p=NS SMTC vs L-NMMA).



4.3.2 Influence of L-NMMA and SMTC on CBF in response to incremental pacing

Incremental pacing did not cause any significant change in blood pressure during saline vehicle in either the L-NMMA (MAP: 98.2±4.46 to 93.3±4.96 mmHg, P=NS)

or SMTC (MAP: 91.9±5.13 to 96.6±6.26 mmHg, P=NS) groups, or during L-NMMA (MAP: 103.6±5.87 to 100.5±6.63 mmHg, P=NS) or SMTC (MAP: 106.6±8.10 to 99.6±5.83 mmHg, P=NS) infusion.

In the presence of saline vehicle, incremental pacing led to an expected increase in CBF in both L-NMMA (from 56.8±9.27 to 83.5±14.2 ml/min; P<0.01) and SMTC (from 67.2±8.98 to 98.5±12.87 ml/min; P<0.01) groups (Figure 4.3). During L-NMMA infusion, although CBF increased in response to incremental pacing (from 45.5±6.76 to 61.6±9.49 ml/min; P<0.01), the magnitude of increase was significantly blunted compared to changes in flow during saline infusion (ΔCBF - L-NMMA: 16.1±3.91ml/min, saline: 26.8±5.74 ml/min; P<0.05 by 2-way ANOVA) (Figure 4.3A and 4.3B). The CBF also increased with pacing during STMC infusion (from 54.7±7.03ml/min to 102.1±16.57 ml/min; P<0.01), but in contrast to L-NMMA, was unchanged from that achieved with saline (102.1±16.6ml/min vs 98.5±12.9 ml/min respectively; P=NS) (Figure 4.3C and 4.3D). Representative coronary Doppler traces demonstrating the effect of L-NMMA on the APV are shown in Figure 4.4.

Figure 4.3: Effect of L-NMMA and SMTC on the CBF response to pacing. L-NMMA vs saline in the coronary blood flow response to pacing plotted against both (A) heart rate and (B) cardiac workload. SMTC vs saline in the coronary blood flow response to pacing plotted against both (C) heart rate and (D) cardiac workload. Sequential atrial pacing in the presence of saline increased CBF in both groups (*P<0.01 both groups). During L-NMMA, CBF increased with pacing (*P<0.01) but was significantly blunted compared to that during saline (ΔCBF L-NMMA vs saline †P<0.05). In patients receiving SMTC, pacing induced increase in CBF (*P<0.01) was similar to that during saline (P=NS).

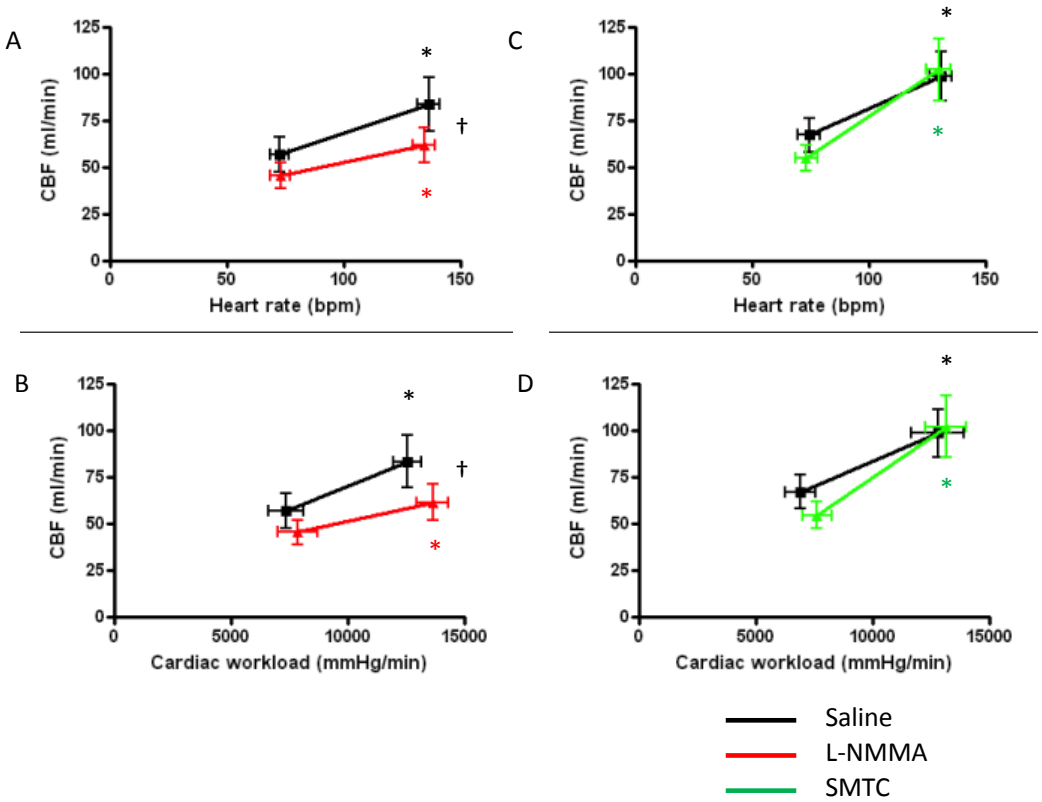
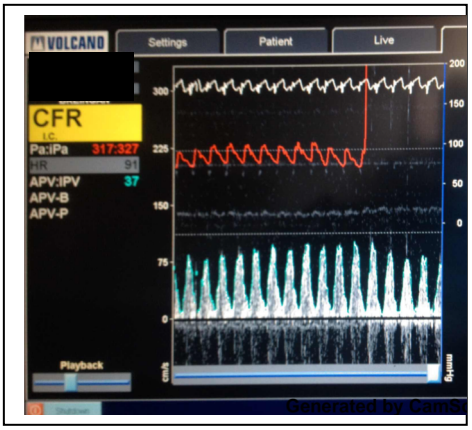


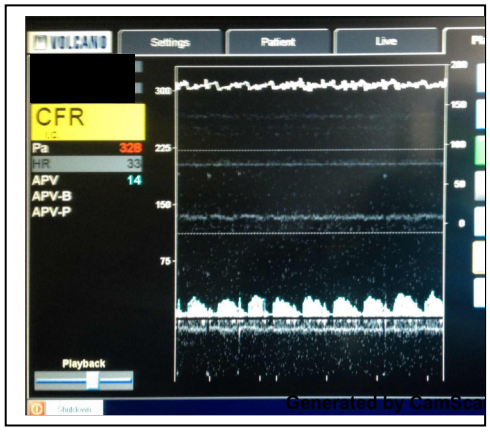
Figure 4.4. Representative coronary Doppler spectral displaying APV (A) at baseline heart rate during saline vehicle (B) at peak heart rate during saline vehicle (C) at baseline heart rate during L-NMMA (D) at peak heart rate during L-NMMA.

A

B



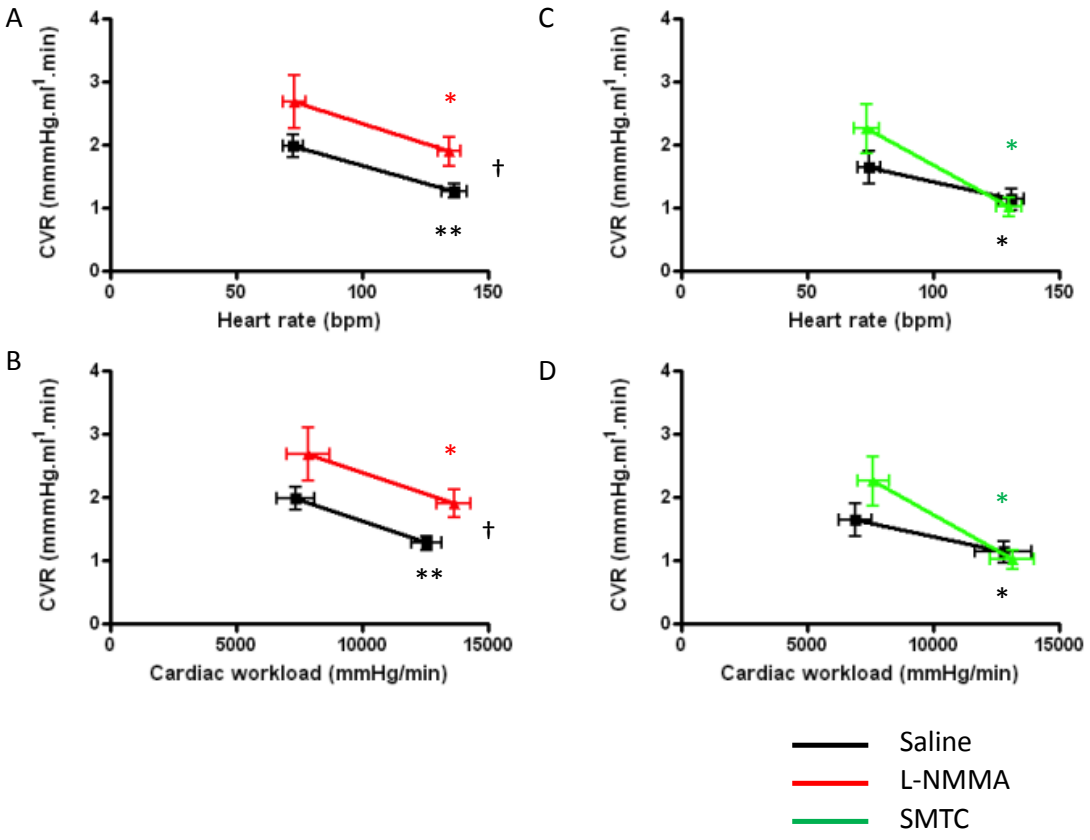
C



D

The effects of pacing on coronary resistance were similar to those on flow. In the presence of saline vehicle, incremental pacing led to a decrease in CVR in both L-NMMA (from 1.98±0.19 to 1.28±0.12 mmHg.ml^{-1}.min; P<0.01) and SMTC (from 1.64±0.27 to 1.14±0.17 mmHg.ml^{-1}.min; P<0.05) groups (Figure 4.5). During L-NMMA infusion, CVR decreased in response to incremental pacing (from 2.68±0.42 to 1.90±0.22 mmHg.ml^{-1}.min; P<0.05) but was significantly greater compared to saline (1.90±0.22 vs 1.28±0.12 mmHg.ml^{-1}.min; P<0.01) (Figure 4.5A and 4.5B). CVR decreased with pacing during STMC infusion (from 2.25±0.40 to 1.02±0.15 mmHg.ml^{-1}.min; P<0.05) but was unchanged from that achieved with saline (1.02±0.15 vs 1.14±0.17 mmHg.ml^{-1}.min; P=NS) (Figure 4.5C and 4.5D).

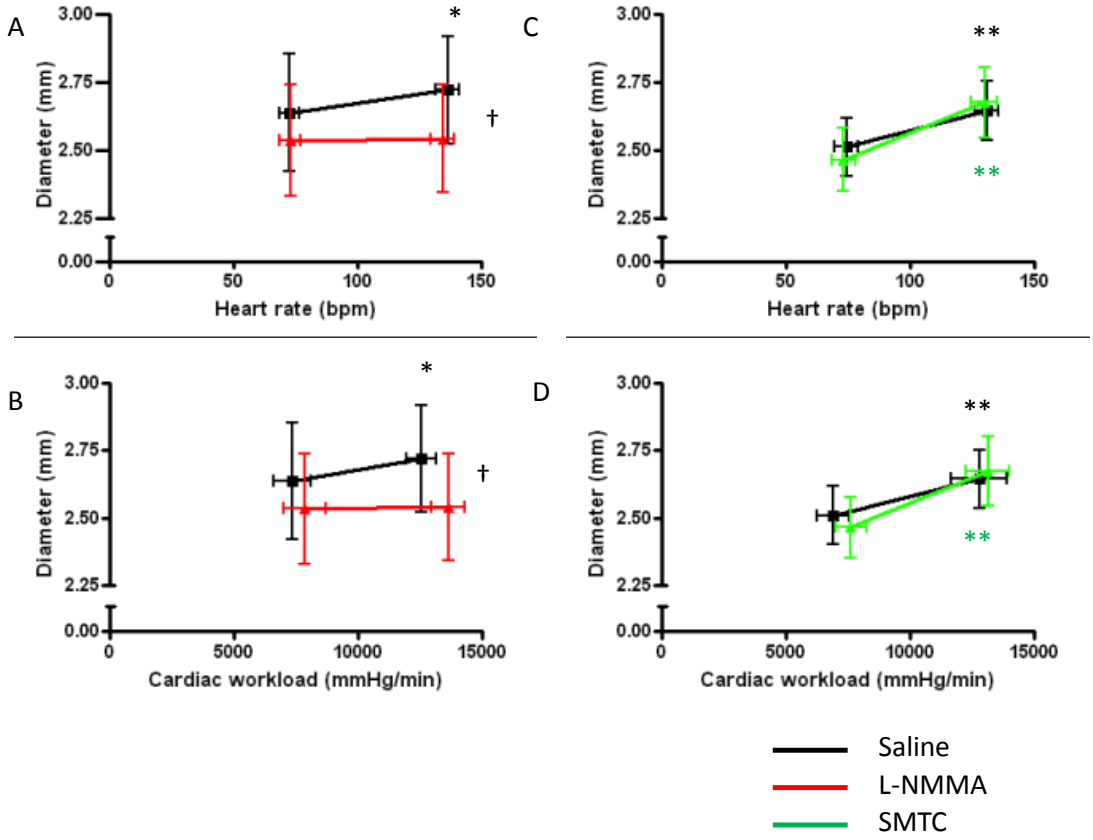
Figure 4.5: Effect of L-NMMA and SMTC on the CVR response to pacing. Influence of L-NMMA vs saline on CVR in response to pacing plotted against both (A) heart rate and (B) cardiac workload. Influence of SMTC vs saline on CVR in response to pacing plotted against both (C) heart rate and (D) cardiac workload. Sequential atrial pacing in the presence of saline decreased CVR in both groups (L-NMMA group: **P<0.01; SMTC group: *P<0.05). During L-NMMA, CVR continued to decrease in response to incremental pacing (*P<0.05) but was significantly greater compared to saline (†P<0.01). In patients receiving SMTC, pacing induced increase in CVR (*P<0.05) was similar to that during saline (P=NS).



4.3.3 Influence of L-NMMA and SMTC on coronary arterial diameter in response to incremental pacing

Sequential atrial pacing in the presence of saline increased coronary arterial diameter in both groups (L-NMMA: 2.64±0.22 to 2.72±0.20 mm; $P<0.05$, and SMTC: 2.51±0.11 to 2.64±0.11 mm; $P<0.01$). This response was blunted with infusion of L-NMMA, where coronary artery diameter did not increase with pacing (2.53±0.20 to 2.54±0.20 mm; P=NS) (Figure 4.6A and 4.6B), but not SMTC, where coronary artery diameter continued to increase in response to atrial pacing (2.46±0.11 to 2.67±0.13 mm; $P<0.001$) (Figure 4.6C and 4.6D).

Figure 4.6: Effect of L-NMMA and SMTC on the coronary diameter response to pacing. Influence of L-NMMA vs saline on coronary artery diameter in response to pacing plotted against both (A) heart rate and (B) cardiac workload. Influence of SMTC vs saline on coronary artery diameter in response to pacing plotted against both (C) heart rate and (D) cardiac workload. Sequential atrial pacing in the presence of saline increased coronary arterial diameter in both groups (L-NMMA group: *P<0.05; SMTC group: **P<0.01). This response was blunted with infusion of L-NMMA (†P<0.001), but not SMTC, where coronary diameter continued to increase in response to atrial pacing (**P<0.01).



4.4 DISCUSSION

The conventional view of NO-dependent regulation of blood flow in humans had been that this mainly involves eNOS in the endothelium of blood vessels. Previous experiments by our group had recently provided evidence that nNOS-derived NO plays a key role in the local regulation of basal blood flow in the human forearm and coronary circulations (Seddon et al., 2009; Seddon et al., 2008). This investigation is the first of its kind to investigate systematically the effects of selective NOS inhibition on the regulation of coronary vasomotor tone during increasing cardiac workload. We found that local infusion of L-NMMA led to a significant reduction in basal CBF, consistent with previous results by our group (Seddon et al., 2009) as well as others using similar doses (Duffy et al., 1999; Egashira et al., 1996; Lefroy et al., 1993; Quyyumi et al., 1995b). SMTC led to a similar reduction in basal CBF to L-NMMA, as previously shown (Seddon et al., 2009), but the novel finding in this study was that during atrial pacing the increase in CBF and coronary artery diameter was blunted by L-NMMA but not so by SMTC.

In animal models, selective inhibition of nNOS or eNOS gene deletion attenuates exercise-induced vasodilatation and increase in blood flow in the skeletal muscle (Fadel et al., 2003; Grange et al., 2001; Kobayashi et al., 2008; Thomas et al., 1998). As previously discussed, in nNOS knockout mice and the *mdx* mouse, the ability of muscle contraction to attenuate α-adrenergic vasoconstriction has been shown to be defective and lead to abnormal flow regulation (Thomas et al., 1998). However, similar results have not been replicated in humans. Continuous short-term adjustments to CBF are fundamentally important to ensure there is a close match between

myocardial tissue perfusion and cardiac workload (Deussen et al., 2012). NO is known to be one of a number of paracrine mediators (including adenosine, prostaglandins, and EDHF's), which in conjunction with vascular autonomic input, determine optimal CBF (Deussen et al., 2012; Garland et al., 2011; Liu et al., 2011).

However, the relative contribution of different NOS isoforms to regulation of epicardial and microvascular tone, and thus blood flow, in response to changes in metabolic demand remains unclear. Our results indicate that the vasoregulatory influences of eNOS and nNOS are complementary to one another, with nNOS primarily influencing CBF at rest, and eNOS contributing to changes in response to increasing metabolic demand as induced through incremental cardiac pacing.

NO has been implicated in regulation of vascular tone in response to changes in metabolic demand in the coronary circulation (Berdeaux et al., 1994; Chi et al., 2003; Duffy et al., 1999; Kuo et al., 1991; Quyyumi et al., 1995a; Tousoulis et al., 1997). Quyyumi and colleagues demonstrated that non-selective inhibition of NOS results in a significant reduction in both microvascular and epicardial vasodilatation during cardiac pacing. In patients with angiographically normal coronary arteries they paced at a mean rate of 140 bpm. During pacing in the control study there was a 50 % increase in CBF and a 9 % increase in coronary artery diameter. During L-NMMA, cardiac pacing produced only a 23 % increase in CBF and the baseline change in diameter remained constant (Quyyumi et al., 1995a). Tousoulis and colleagues again found no increase in coronary artery diameter during L-NMMA whilst pacing at 140 bpm in angiographically normal arteries, with significantly reduced change in CBF during pacing (Tousoulis et al., 1997).

Increased myocardial oxygen demand leads to metabolic dilatation of the small resistance coronary arterioles to augment local blood flow and oxygen supply to match demands (Macho et al., 1981). As a consequence of this microvascular dilatation, dilatation of large epicardial arteries follows due to increased flow and hence shear stress. This FMD response can be induced by physiological stimuli (such as dynamic exercise or atrial pacing) and is considered an important regulatory mechanism in the coronary circulation in humans. During pacing, we confirmed previous findings that angiographically normal human coronary arteries dilate and CBF increases (Gaglione et al., 1987; Gordon et al., 1989; Nabel et al., 1990a; Quyyumi et al., 1995a). Pacing-induced FMD in the coronary arteries is abolished by L-NMMA (Duffy et al., 1999; Quyyumi et al., 1995a; Tousoulis et al., 1997), confirming the role of NO in this process. In addition, atherosclerosis, which is known to reduce local bioavailability of NO, blunts CBF and/or epicardial dilatation in response to a host of different metabolic stimuli such as bicycle exercise, cold pressor, mental stress as well as pacing (Gordon et al., 1989; Nabel et al., 1990a, b; Yeung et al., 1991)

Previous studies have shown that SMTC had no effect on the increase in CBF or conduit epicardial artery dilatation induced by intracoronary substance P. In contrast, L-NMMA significantly inhibited the response to substance P (Seddon et al., 2009). The effect of substance P is likely to represent the combination of a direct effect on the epicardial artery (Toda and Okamura, 1989) and a result of FMD. This is similar to the response in the forearm vasculature, where SMTC reduced basal tone but had no effect on the increase in forearm blood flow induced by the agonist, ACh, or shear stress by FMD, whereas these were inhibited by L-NMMA (Seddon et al., 2009; Seddon et al.,

2008). Peripheral vasomotor function has been shown to be related to vasomotor function in the coronary arteries in individuals (Anderson et al., 1995), despite peripheral and myocardial circulations differing in terms of microvascular architecture, metabolic regulation, patterns of blood flow and vascular resistance, and the pathways that are activated to induce hyperaemia (Komaru et al., 2000).

Although previous studies have already established the role of NO in regulation of CBF during changes in metabolic demand, none as in our study, has directly examined the relative contribution of eNOS- and nNOS-derived NO to this regulatory process. Our results further indicate that NOS inhibition significantly attenuates, but does not abolish changes in CBF. This confirms the notion that pathways controlling metabolic regulation of blood flow have significant redundancy through interdependence on a host of metabolites including NO, such as adenosine, prostaglandins, as well as neural inputs (Deussen et al., 2012).

Our study did not examine the association between nNOS and autonomic mediated changes in metabolic demand in the heart as seen during exercise or mental stress. Human studies have shown that mental stress-induced vasodilatation in the forearm is significantly attenuated by selective inhibition of nNOS. However, this response is not influenced by a vasoconstrictor (norepinephrine) that reduces basal flow to a similar extent to the NOS inhibitor. Taken together these findings strongly suggest a role for local nNOS-derived NO in regulation of microvascular tone during mental stress (Seddon et al., 2008). On the basis of the emerging evidence future studies should also examine the role of nNOS in regulation of CBF in response to autonomic stimulation. In addition, at a cellular level nNOS has been isolated to both the peri-vascular

autonomic fibres as well as the vessel wall (Bachetti et al., 2004; Boulanger et al., 1998; Schuman and Madison, 1994).

Animal studies have indicated that there may be an inverse functional association between different NOS isoforms in the vascular wall where a reduction in eNOS expression is compensated for by an increase in nNOS activity (Boulanger et al., 1998). This is an important association which requires further exploration in humans in particular to identify how presence of conventional cardiovascular risk factors and/or atherosclerosis may influence simultaneous nNOS / eNOS expression and hence CBF during episodes of increased metabolic demand.

4.4.1 Study limitations

Pacing-induced change in CBF is not necessarily a pure model of increased workload and may also be influenced by other factors such as cardiac mechanics and the duration of diastole. Nevertheless, it is an objective and easy-to-implement intervention in the invasive setting of a clinical cardiac catheterization laboratory.

It should also be noted that these studies were not performed in healthy volunteers and that most of the patients who took part in these studies had at least 1 risk factor for coronary disease, which could have potentially influenced the results. Such patients have been demonstrated to have impaired vasomotor responses (Quyyumi et al., 1995b; Quyyumi et al., 1997; Vita et al., 1990). Consequently, although all the patients had angiographically normal coronary arteries, it is unlikely that this represented completely normal vasomotor function.

The baseline characteristics of the two groups given either SMTC or L-NMMA were well matched, there were a mixture of males and females, and the total number of subjects studied was small. Related to this, and due to the fact that in the SMTC group the generally smaller circumflex artery was studied in slightly more subjects, due to either difficulty placing the wire in the LAD or due to LAD vessels with unsuitable anatomy for precise measurement (e.g. tortuosity), the baseline mean epicardial conduit artery diameter was slightly larger in the L-NMMA group.

4.4.2 Conclusion

Our study provides the first direct evidence that increases in CBF in response to pacing-induced change in metabolic demand is mediated by eNOS- as opposed to nNOS-derived NO, whilst confirming the latter plays an important role in setting basal coronary resistance.

CHAPTER 5:

FIRST-IN-MAN STUDY OF THE EFFECTS OF nNOS INHIBITION ON SYSTEMIC HAEMODYNAMICS

5.1. INTRODUCTION

It has been well documented in both animal and human studies that non-selective NOS inhibition with L-NMMA causes a transient pressor response when infused systemically and this has been assumed to be mediated through inhibition of eNOS (Gamboa et al., 2007; Hansen et al., 1994a; Haynes et al., 1993; Owlya et al., 1997; Stamler et al., 1994). In our own lab, Brett and colleagues infused L-NMMA systemically in men and observed modest transient increases (compared with effect of saline placebo) in systolic and diastolic blood pressures of 4.1±1.1 % and 12.6±3.5 % respectively, suggesting that normal arterial blood pressure is kept down by the continuous generation of NO (Brett et al., 1998). This NO could be generated at different sites that regulate blood pressure, such as resistance vessels, the brain and the kidneys.

As discussed in this thesis, recent evidence suggests that a major source of basal NO release regulating local vascular tone in humans is nNOS (Seddon et al., 2009; Seddon et al., 2008). Local nNOS-selective inhibition with SMTC reduces basal blood flow in

the forearm and coronary circulations without affecting endothelial-mediated vasodilatation. These findings suggest that, through regulation of local vascular tone, nNOS may influence SVR and hence blood pressure. In addition, nNOS in the brain, kidneys and myocardium could also affect blood pressure. This study aimed to examine the hypothesis that nNOS systemically regulates blood pressure in man using the specific nNOS inhibitor SMTC and measuring changes in CO, blood pressure and SVR. This would establish if some or all of the effects previously attributed to eNOS are in fact due to nNOS. SMTC had been given locally via the intra-brachial and intra-coronary routes but not in doses that would have a systemic effect. Hence this would be a first-in-man study of the effects of systemic nNOS-specific inhibition on haemodynamics.

5.1.1 Study aims

The aim of the proposed study was to establish the effects of acute systemic inhibition of nNOS with SMTC on the regulation of systemic haemodynamics, i.e. SVR, CO, and BP, for the first time in humans.

5.2 METHODS

The studies were approved by the local research ethics committee (St.Thomas' Hospital). Healthy male volunteers were recruited from a list of suitable subjects who had participated in previous departmental studies, as described in chapter 2.

5.2.1 SMTC Dosing

SMTC 0.2 μmol/min produces a similar reduction in FBF to that achieved by LNMMA 2 μmol/min (Seddon et al 2008) and is therefore approximately 10 times more potent than LNMMA to increase forearm resistance. Hence, we assumed that the dose of SMTC required to achieve systemic effects would be approximately 10 times more potent than L-NMMA. We wanted to establish a dose of SMTC which produces a 30 to 40 % increase in SVR as this is what is observed with similar studies using systemic L-NMMA. L-NMMA at 12-24 μmol/Kg (3-6 mg/Kg) (bolus dose over 5 min) gives a 20-40 % increase in SVR, an effect which is maximal within 10 to 15 min after the start of the infusion (Brett et al., 1998; Haynes et al., 1993). We therefore expected a SMTC dose of 1.2-2.4 μmol/Kg to produce the same increase in SVR.

We calculated the total SMTC dose that we had already given in forearm and coronary studies to establish a safe starting point for a rising single dose study. The maximum dose administered in forearm studies is 0.2 μmol/min x ~20 min study = 4 μmol. Maximum dose in coronary studies is 0.625 μmol/min x ~12 min study = 7.5 μmol. We chose a starting dose which would be similar to the dose that we had already given, and hence started with 0.1 μmol/Kg to be given as a bolus over 10 min, which

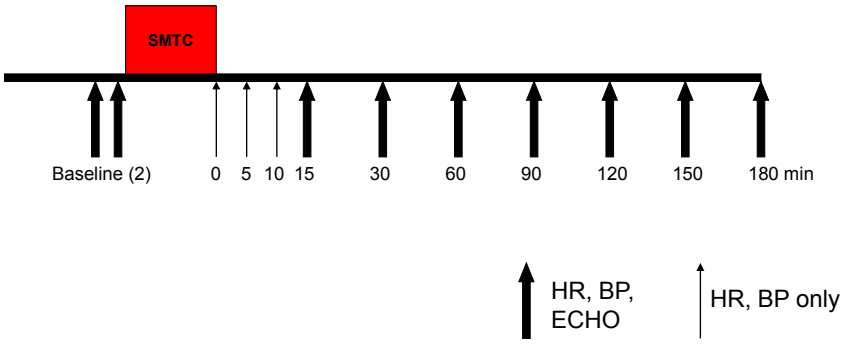
for an average 70 Kg man would be a total systemic dose of 7 μmol. Doses were first administered in a rising single dose study and the regimen for the study was SMTC 0.1, 0.3, 1.0, 3.0 μmol/Kg and placebo on separate occasions (a subject would attend on five separate occasions), with placebo randomised in relation to the rising dose schedule, stopping when we established a dose with a 30-40 % increase in SVR.

5.2.2 Protocol 1: dose response study

Participants were asked to abstain from caffeine for at least 12 hours before the studies. Subjects attended study days at least 1 week apart. The subjects also attended for safety blood checks (FBC, renal and liver profiles) 3 to 5 days after the study.

Studies were undertaken in a quiet temperature-controlled vascular laboratory (23 °C to 25 °C) after at least 30 min of rest. A 16-gauge intravenous cannula was inserted into the ante-cubital fossa. An ECG was recorded and then baseline measurements were taken. HR and BP were measured using a standard oscillometric method (Omron 705CP) and according to established guidelines (O'Brien et al., 2003), and left ventricular SV was measured using 2D trans-thoracic echocardiography as described in chapter 2. CO was calculated as SV multiplied by the HR, and SVR was calculated as MAP/CO. SMTC (0.1, 0.3, 1.0, and 3.0 μmol/Kg), in rising doses according to visit, was given by intravenous infusion over 5 min. HR and BP was recorded at 0, 5 and 10 min after completion of SMTC infusion. At 15 min HR, BP and SV were measured. All these measurements were repeated at 30 min after infusion, and then every 30 min for a total of 3 hours to ensure values had returned to baseline. At the end of the study an ECG was recorded (Figure 5.1).

Figure 5.1: Schematic of study protocol. Measurement were made at baseline and then after infusion of IV SMTC.



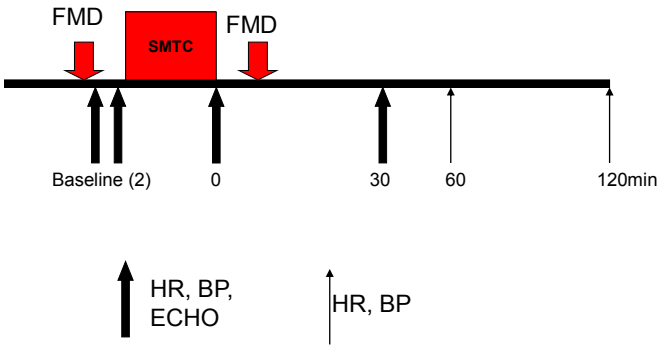
5.2.3 Protocol 2: randomised crossover study

The protocol was altered to undertake a randomised crossover study with blinded haemodynamic assessment in which the highest dose of SMTC achieved from protocol 1 or saline vehicle were infused on separate occasions (2 visits per subject) (Figure 5.6). The dose of SMTC used was the dose found to produce an increase in SVR of about 30-40 %. BP and HR were measured by an oscillometric method as before and SV was measured using 3D echocardiography to allow for more accurate assessment, as described in chapter 2.

To assess whether this dose of SMTC was affecting eNOS, brachial FMD was measured, as described in chapter 2. Again, CO was calculated as the SV multiplied by the HR, and SVR as MAP/CO. Measurement of HR, BP and SV were made at

baseline and then at 0 min and 30 min after the end of infusion. FMD was measured at baseline and then again after SMTC infusion, immediately after HR, BP and echocardiography measurements at 0 min were completed.

Figure 5.2: Schematic of protocol 2.



5.2.4 Statistical analysis

Data were summarized as mean ± standard error of the mean (s.e.m.). Changes in HR and BP were expressed as the absolute changes in bpm and mmHg from baseline respectively. Changes in SV, CO and SVR were expressed as percentage changes from baseline. Effects of SMTC on the haemodynamic responses were analysed by paired t-tests or ANOVA for repeated measures as appropriate. All tests were two-tailed and differences were considered significant when $P<0.05$.

5.3 RESULTS

5.3.1 Protocol 1: dose response study

Nine healthy male subjects aged 21.1±0.87 years were studied. The baseline characteristics are shown in table 5.1.

Table 5.1: Baseline characteristics of the subjects in protocol 1 (dose response study).

Healthy male volunteers (n=9)	
Age (years)	21.1±0.87
Systolic blood pressure (mmHg)	127.3±2.87
Diastolic blood pressure (mmHg)	74.2±2.98
Height (m)	1.80±0.02
Weight (kg)	77.8±4.65
Body Mass Index (kg/m^2)	23.8±1.06

No subjects had any changes in biochemical or haematological profiles after infusion of SMTC. The SMTC had to be infused over ~10 min rather than 5 min due to mild irritation felt by a few subjects during infusion, especially at the higher doses (due to the pH of the SMTC solution which was lower with each increase in dose). This

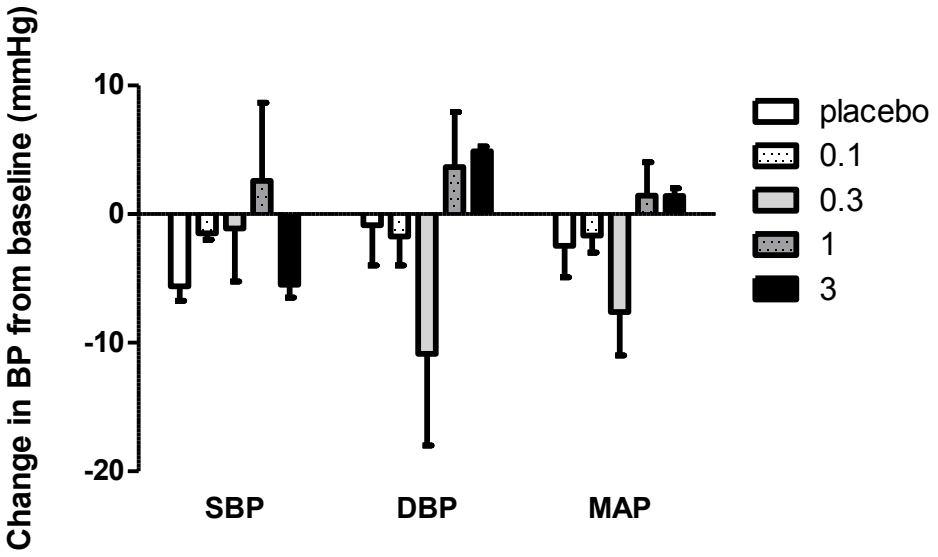
irritation would cease as soon as the SMTC was stopped. The longer infusion time and using a large vein in the ante-cubital fossa resolved this issue. There were no other adverse effects.

After the first 3 subjects we analysed the data to assess whether the SMTC was having any effect at the doses studied. The first 3 subjects received SMTC 0.1, 0.3, 1.0 and 3.0 μmol/Kg and placebo. Unfortunately one of the subjects withdrew from the study before completion (personal reasons not related to the study) and only received SMTC 0.1, 0.3, 1.0 μmol/Kg (did not receive SMTC 3.0μmol/Kg or placebo).

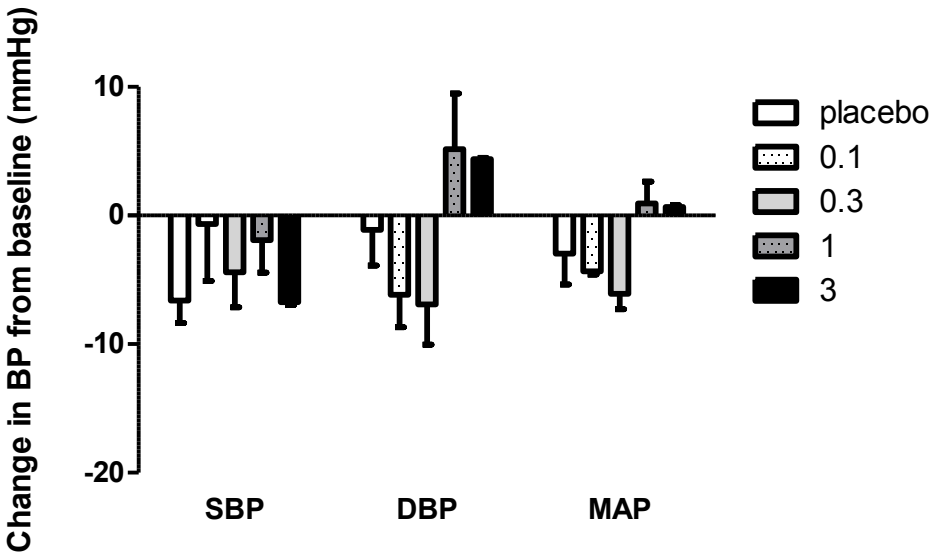
The 2 lower doses of SMTC (0.1 and 0.3μmol/Kg) did not appear to affect BP, whereas the higher doses (1.0 and 3.0μmol/Kg) did, especially DBP and MAP (Figure 5.2). This was expected to be the case as the lowest dose of SMTC was equivalent to what has been given in the coronary studies and had no effect on BP. For the next 6 subjects the dosing schedule was changed to SMTC 1.0, 3.0 μmol/Kg and placebo (3 visits per subject), with placebo randomised in relation to the rising dose schedule.

Figure 5.3: Review after 3 subjects. Change in SBP, DBP and MAP at different doses of SMTC (placebo, 0.1, 0.3, 1.0, 3.0 μmol/Kg) for the first 3 subjects at (A) 0 min (B) 5 min (C) 10 min (D) 15 min after the end of infusion. There appears to be an increase in DBP and MAP at the higher doses of SMTC, which appears greater the earlier it is after the completion of SMTC infusion.

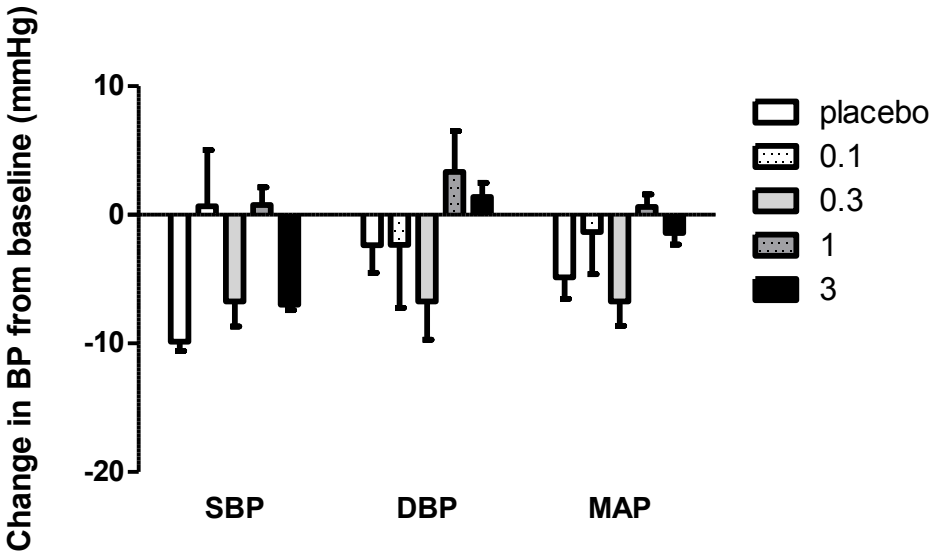
(A) 0 min after end of infusion



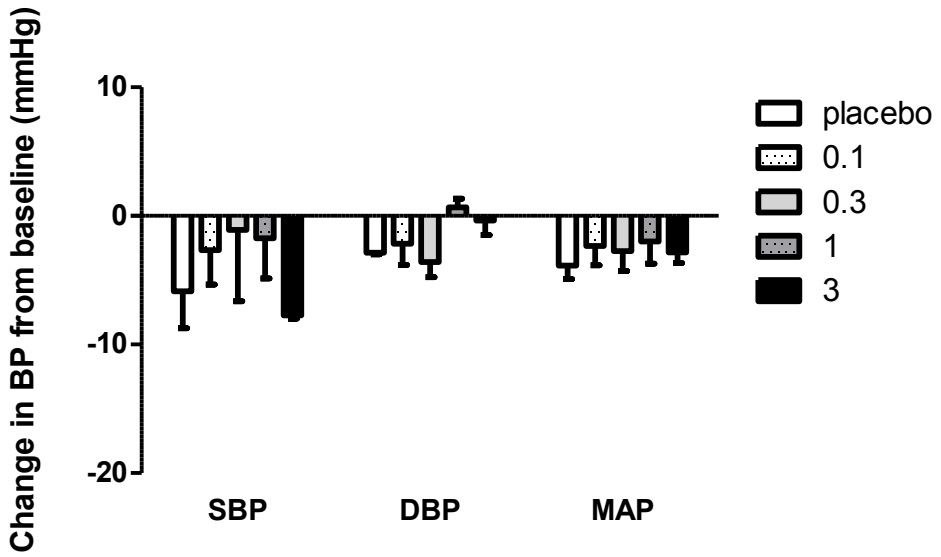
(B) 5 min after end of infusion



(C) 10 min after end of infusion



(D) 15 min after end of infusion



A total of 9 subjects therefore had SMTC infused at 1.0 and 3.0 μmol/Kg. No subjects had any changes in biochemical or haematological profiles after infusion of SMTC. The SMTC was infused over ~10 min through a large vein in the ante-cubital fossa to minimise local irritation. There were no other adverse effects.

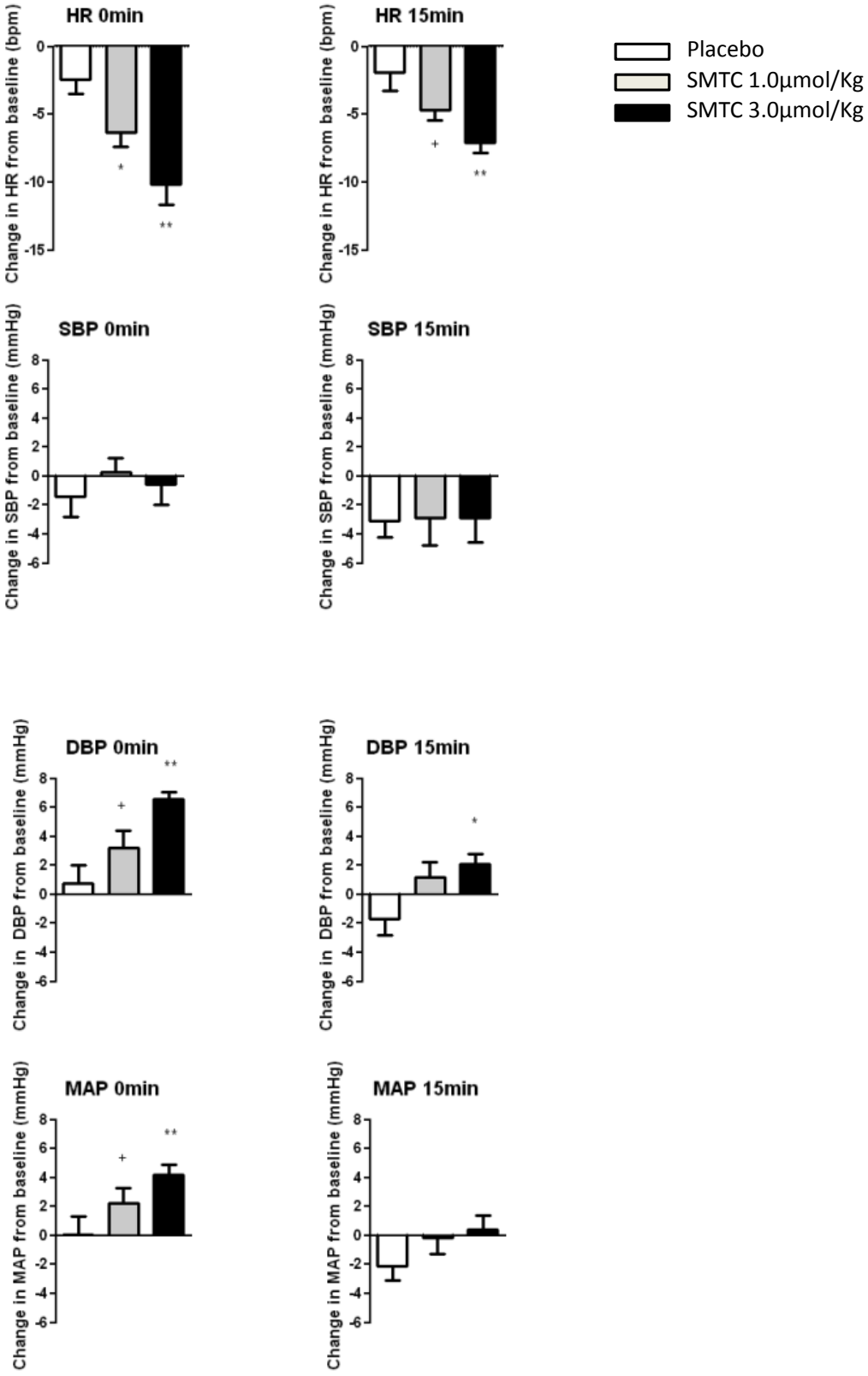
When compared to placebo, SMTC significantly decreased HR, increased DBP and MAP, decreased SV and CO, and increased SVR from baseline in a dose-dependent manner. There was no effect on SBP. The maximum effect on HR and BP was immediately after the end of SMTC infusion, i.e. 0 min (Figure 5.4). At 0 min, when compared to placebo, SMTC 3.0 μmol/Kg significantly decreased HR from baseline by 7.72±1.46 bpm (-10.2±1.37 during SMTC vs -2.47±0.94 bpm during placebo; n=8, $P<0.01$), increased DBP by 5.78±1.13 mmHg (6.56±0.44 vs 0.78±1.08 mmHg; n=8, $P<0.01$) and increased MAP by 4.11±1.05 mmHg (4.18±0.61 vs 0.06±1.09 mmHg;

n=8, P<0.01). There was no change in SBP (-0.59±1.21 vs -1.38±1.29 mmHg; n=8, P=NS).

Figure 5.4 (next page): The effect of SMTC 1.0 and 3.0 μmol/Kg on HR, SBP, DBP and MAP at (A) 0 min (B) 15 min after the end of infusion. SMTC increases HR, decreases DBP and decreases MAP in a dose-dependent manner, with greater effect earlier after the end of the infusion. *P<0.05 for HR at 0 min SMTC 1.0 μmol/Kg vs placebo; and for DBP at 15 min SMTC 3.0 μmol/Kg vs placebo. **P<0.01 for HR at 0 and 15 min SMTC 3.0 μmol/Kg vs placebo; for DBP at 0min SMTC 3.0 μmol/Kg vs placebo; and MAP at 0 min SMTC 3.0 μ mol/Kg vs placebo.

+P<0.05 for HR at 15 min SMTC 3.0 μmol/Kg vs SMTC 1.0μmol/Kg; for DBP at 0min SMTC 3.0 μmol/Kg vs SMTC 1.0 μmol/Kg; and for MAP at 0 min SMTC 3.0 μmol/Kg vs SMTC 1.0 μmol/Kg.

(A) (B)


HR 0min
HR 15min
Placebo
SMTC 1.0µmol/Kg
SMTC 3.0µmol/Kg
Change in HR from baseline (bpm)
SBP 0min
SBP 15min
Change in SBP from baseline (mmHg)
DBP 0min
DBP 15min
Change in DBP from baseline (mmHg)
MAP 0min
MAP 15min
Change in MAP from baseline (mmHg)

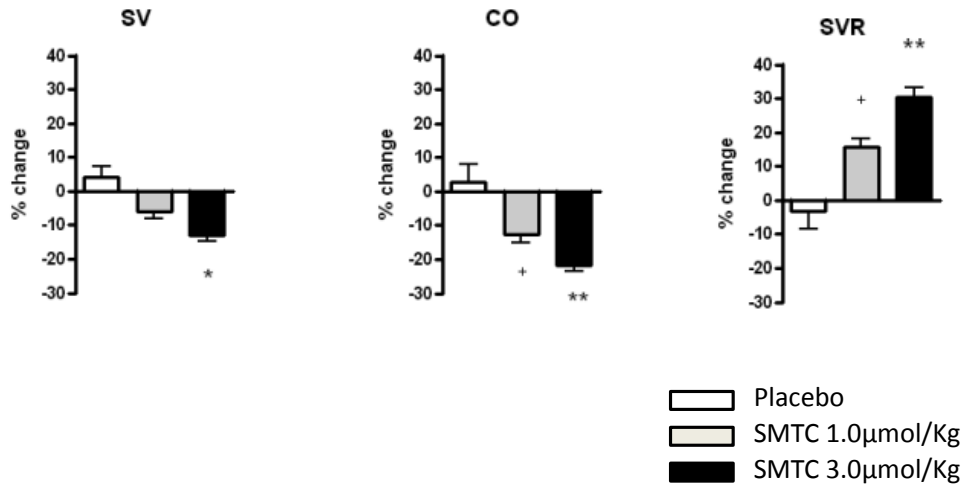
At 15min post SMTC, the first set of readings were taken of SV using 2D echocardiography. This was only carried out on the final 6 subjects in this study. In one subject the echocardiogram for the visit in which SMTC 1 μmol/Kg was infused could not be analysed due to technical reasons. When compared to placebo, SMTC 3.0 μmol/Kg significantly decreased SV by 17.2±4.40 % (-13.1±1.85 vs 4.14±3.18 %; n=6, $P<0.05$), decreased CO by 24.7±5.99 % (-21.9±1.70 vs 2.86±5.16 %; n=6, $P<0.01$) and increased SVR by 33.8±6.28 % (30.5±2.65 vs -3.28±5.27 %; n=6, $P<0.01$) (Figure 5.5).

Figure 5.5: The effect of SMTC 1.0 and 3.0 µmol/Kg on SV, CO and SVR at 15 min after the end of infusion. SMTC significantly decreased SV, decreased CO, and increased SVR in a dose-dependent manner.

*P<0.05 for SV SMTC 3.0 µmol/Kg vs placebo.

**P<0.01 CO and SVR SMTC 3.0 µmol/Kg vs placebo.

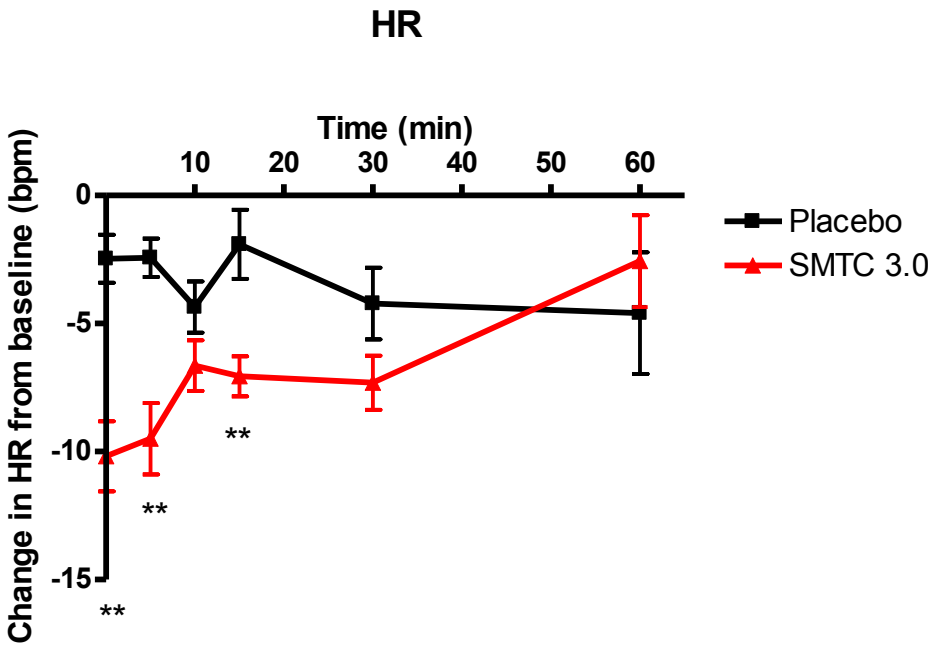
+P<0.05 CO and SVR SMTC 3.0 µmol/Kg vs SMTC 1.0 µmol/Kg.



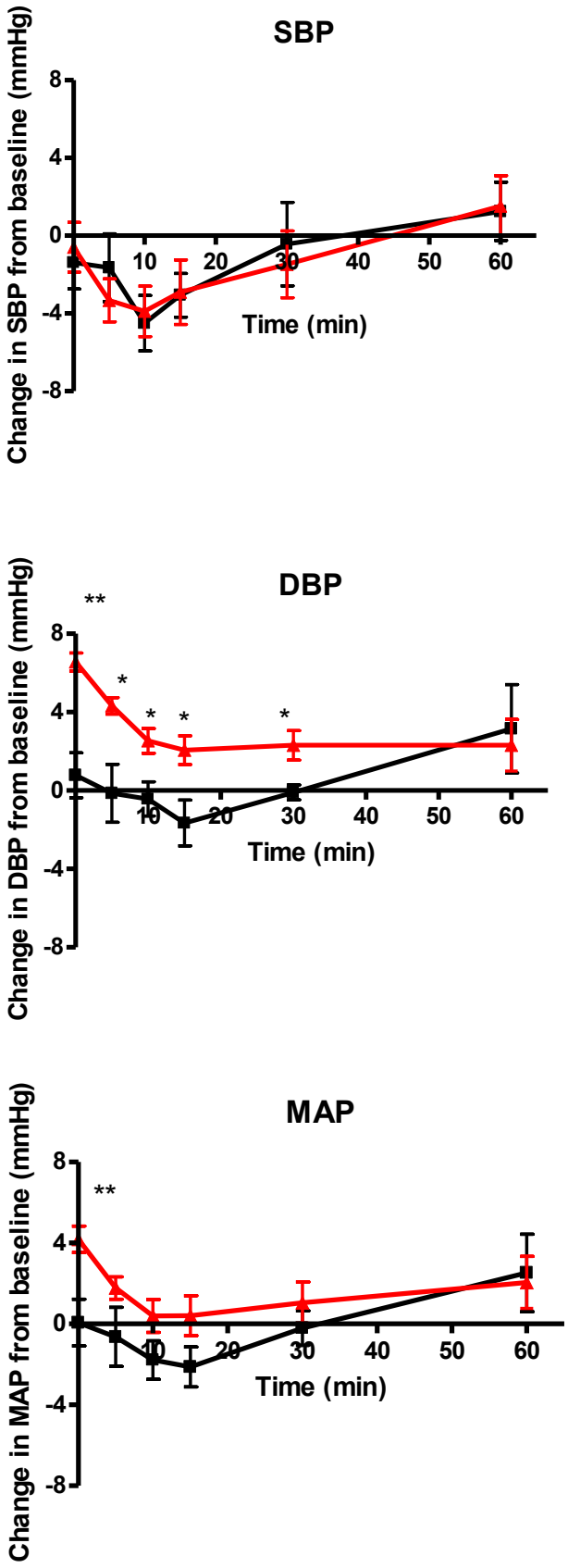
The effect of SMTC decreased with time and at 60 min after the end of the infusion, SMTC 3.0 µmol/Kg no longer had an effect on any of the haemodynamic parameters when compared to placebo (Figure 5.6).

Figure 5.6: The effect of SMTC 3.0 µmol/Kg 0 to 60 min after the end of infusion for (A) HR (B) BP (C) SV and CO (D) SVR. By 60 min SMTC 3.0 µmol/Kg no longer had any effect on any of the systemic haemodynamics when compared to placebo. **P<0.01, *P<0.05 (SMTC 3.0 µmol/Kg vs placebo).

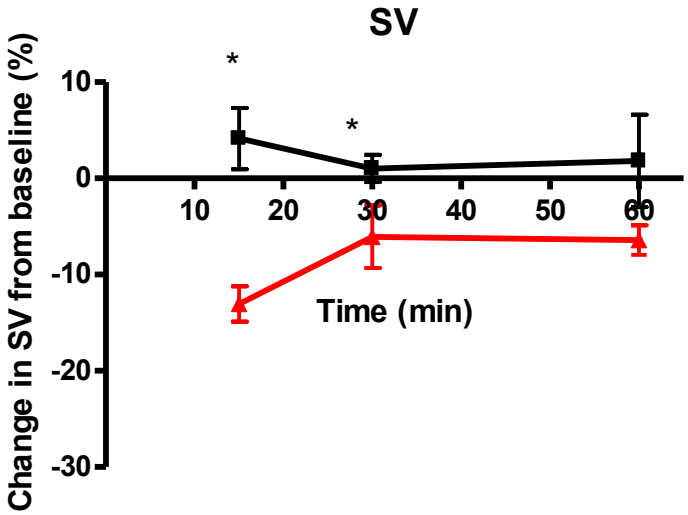
(A)

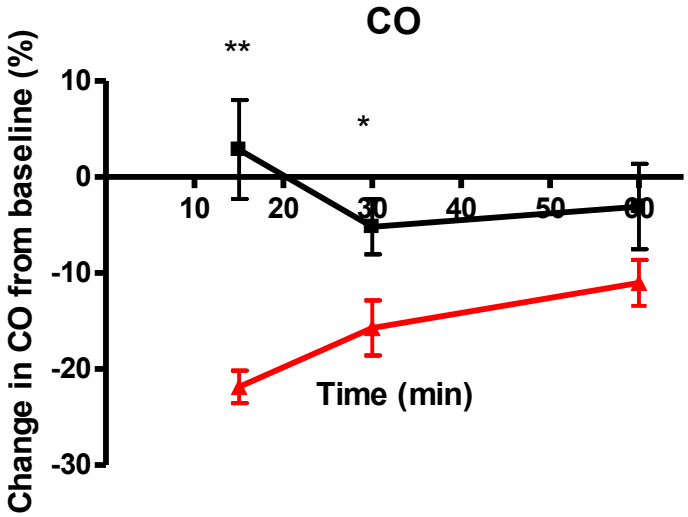


(B)

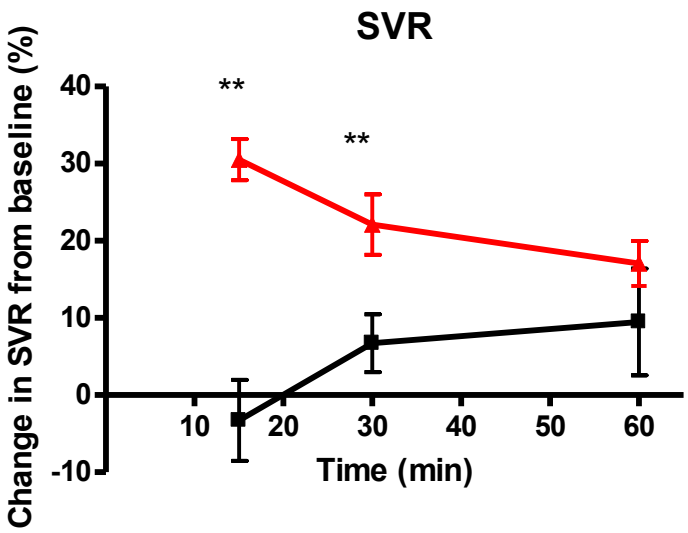

SBP
Change in SBP from baseline (mmHg)
8
4
0
-4
-8
10
20
30
40
50
60
Time (min)
DBP
Change in DBP from baseline (mmHg)
**
*
*
*
*
8
4
0
-4
-8
10
20
30
40
50
60
Time (min)
MAP
Change in MAP from baseline (mmHg)
**
8
4
0
-4
-8
10
20
30
40
50
60
Time (min)

(C)


SV
Change in SV from baseline (%)
10
0
-10
-20
-30
10
20
30
40
50
60
*
*
Time (min)


CO
Change in CO from baseline (%)
10
0
-10
-20
-30
10
20
30
40
50
60
**
*
Time (min)

(D)



5.3.2 Protocol 2: randomised crossover study

Eight healthy male subjects aged 27.8±2.22 years were studied. The baseline characteristics are shown in table 5.2. The above results suggested that the peak action of SMTC is at 0 min after the end of infusion rather than at 10-15 min. This likely represents the longer infusion time of the SMTC when compared to studies using L-NMMA, usually given over 3-5 min and producing a maximum effect at ~15 min after the start of the infusion (Brett et al., 1998). The protocol was altered for the next 8 subjects and we undertook a randomised crossover study with blinded haemodynamic assessment in which the highest dose of SMTC (3.0 μmol/Kg over 10 min) or saline vehicle was infused on separate occasions (2 visits per subject) (Figure 5.2). The dose of SMTC used was the dose found in protocol 1 to produce an increase in SVR of about 30-40%. FMD was measured at baseline and then again after SMTC infusion, immediately after HR, BP and echocardiography measurements at 0 min were

completed. This was at about 10-15 min after the end of infusion, which from the results in protocol 1 showed that at this point SMTC 3.0 μmol/Kg increased SVR by ~34% from baseline when compared to placebo. Blood samples for SMTC concentration were also collected from the subjects. These were taken at baseline (before SMTC infusion), at 20 min after SMTC (immediately after all measurements including FMD had been completed), and at 60 min after SMTC. These samples were sent to a mass spectrometry facilty for measurement of SMTC concentration.

Table 5.2: Baseline characteristics of the subjects in protocol 2 (randomised crossover study).

Healthy male volunteers (n=8)	
Age (years)	27.8±2.22
Systolic blood pressure (mmHg)	128.8±2.40
Diastolic blood pressure (mmHg)	77.9±2.86
Height (m)	1.77±0.02
Weight (kg)	73.1±3.81
Body Mass Index (kg/m^2)	23.3±0.99

There were no changes in haematological or biochemical profiles and no adverse reactions. All 8 subjects had HR, BP and FMD data (n=8) but for one subject the echocardiography data could not be used due to technical reasons (n=7). Left ventricular stroke work (SW) was also calculated, as SV multiplied by MAP.

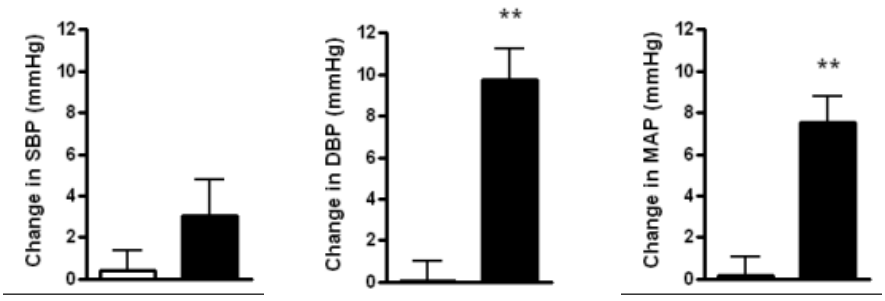
5.3.2.1 Time point: 0 min after end of infusion

When compared to placebo, SMTC 3.0 μmol/Kg reduced HR by 6.10±1.38 bpm (-9.16±1.29 vs -3.06±0.72 bpm; n=8, P<0.01), increased DBP by 9.72±1.78 mmHg (9.75±1.52 vs 0.03±0.95 mmHg; n=8, P<0.001), increased MAP by 7.37±1.70 mmHg (7.52±1.25mmHg vs 0.15±0.91 mmHg ; n=8, P<0.01), decreased SV by 14.0±3.23% (-6.78±5.63 vs 7.19±4.20 %; n=7, P<0.01), reduced CO by 22.8±2.5 % (-21.4±5.94 vs 1.41±4.34 %; n=7, P<0.001), and increased SVR by 42.2±6.4 % (42.3±10.1 vs 0.10±4.84 %; n=7, P<0.001). SMTC did not significantly change SBP (3.06±1.79 vs 0.38±0.98 mmHg; n=8, P=NS) or SW (1.57±6.64 vs 7.44±3.78 %; n=7, P=NS) (Figure 5.7).

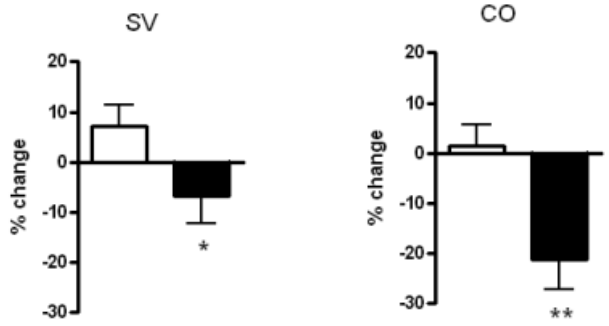
Figure 5.7: The effect of SMTC 3.0 µmol/Kg in study 2 at 0 min after the end of infusion, on (A) HR (B) BP (C) SV and CO (D) SVR. SMTC decreased HR, SV and CO; and increased DBP, MAP and SVR. SMTC did not affect SBP. *P<0.01, **P<0.001 (SMTC 3.0 µmol/Kg vs placebo).

(A)

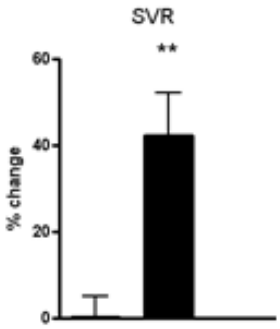
(B)


Change in SBP (mmHg)
Change in DBP (mmHg)
Change in MAP (mmHg)
**
**
0
2
4
6
8
10
12

(C)

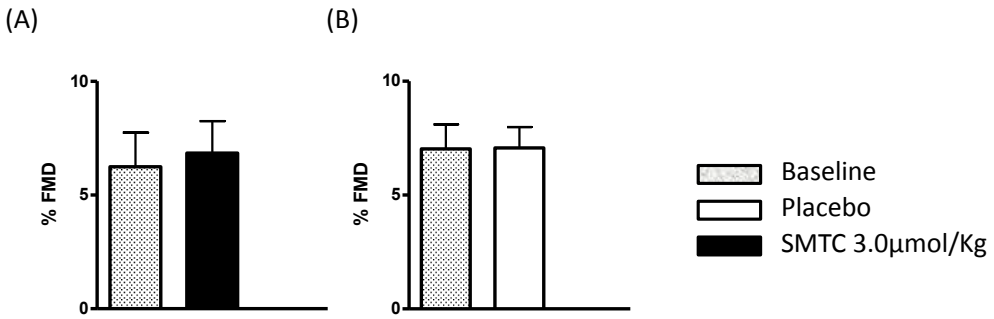

SV
CO
% change
% change
*
**
20
10
0
-10
-20
-30

(D)



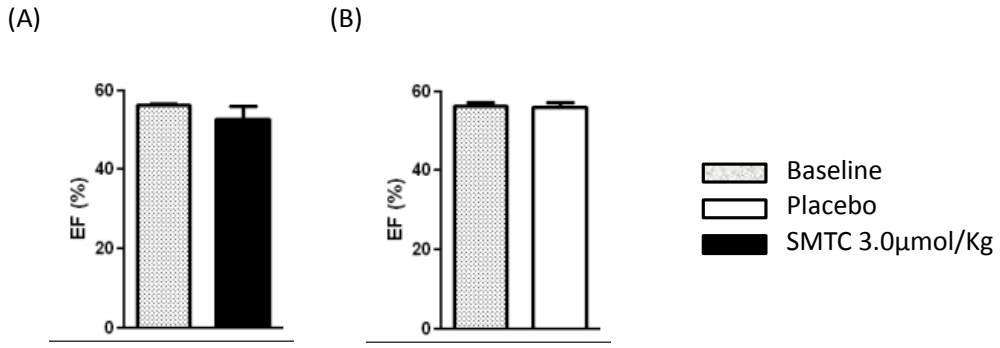
SMTC 3.0 µmol/Kg had no significant affect on FMD (6.24±1.50 to 6.84±1.41 %; n=8, P=NS). In the placebo study FMD did not change either (7.03±1.07 to 7.07±0.91 %; n=8, P=NS) (Figure 5.8).

Figure 5.8: The effect of SMTC 3.0 µmol/Kg on FMD. (A) SMTC did not affect FMD. (B) FMD did not change during placebo.



Using 3D echocardiography we also calculated the left ventricular EF. SMTC 3.0 µmol/Kg had no significant affect on EF, which did not change in the placebo study either. There was also no difference in the change in EF when comparing SMTC to placebo (SMTC: 56.2±0.31 to 52.7±3.29 %, Placebo: 56.2±1.10 to 55.9±1.16 %, n=7, P=NS for both; ΔEF SMTC vs placebo, n=7, P=NS) (Figure 5.9).

Figure 5.9: The effect of SMTC 3.0 µmol/Kg on left ventricular ejection fraction. (A) SMTC did not affect EF. (B) EF did not change during placebo.



5.3.2.2 Time point: 30 min after end of infusion

At 30 min after SMTC infusion, when compared to placebo, SMTC no longer had an effect on HR (-2.84±1.25 vs -2.88±1.40 bpm; n=8, P=NS), SBP (0.50±1.64 vs -0.88±1.23 mmHg; n=8, P=NS), DBP (2.31±1.85 vs 3.34±1.08 mmHg; n=8, P=NS) or MAP (1.71±1.65 vs 1.94±0.88 mmHg; n=8, P=NS). Although not statistically significant, there was a trend suggesting that when compared to placebo, SMTC still reduced SV by 16.13±10.39 % (0.11±5.94 vs 16.3±6.72 %; n=7, P=NS), reduced CO by 16.6±10.6 % (-5.60±6.30 vs 10.9±6.42 %; n=7, P=NS), and increased SVR by 15.8±10.8 % (10.3±7.82 vs -5.49±5.08 %; n=7, P=NS).

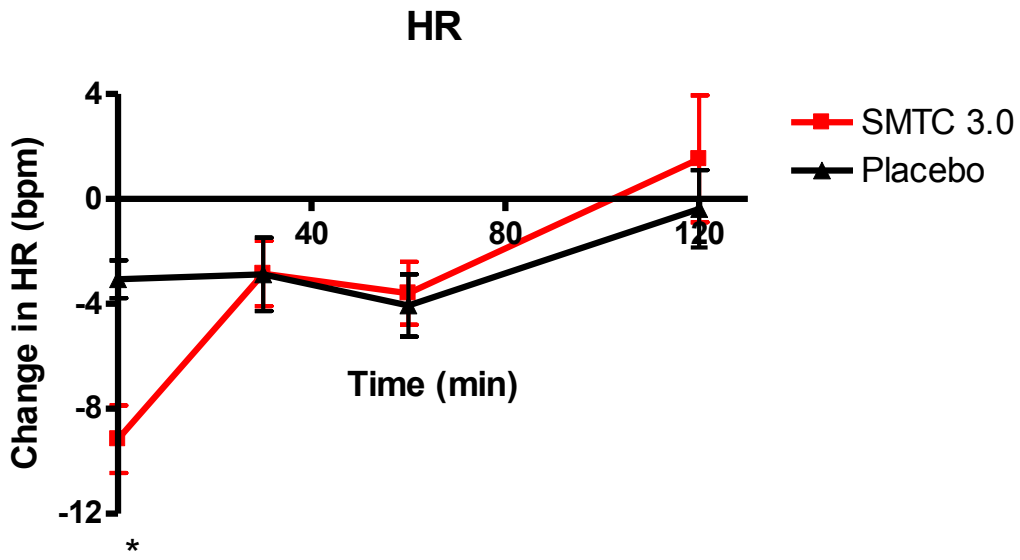
When compared to placebo, SMTC did not significantly change EF (SMTC: 56.2±0.31 to 57.6±2.79 %, Placebo: 56.2±1.10 to 57.5±2.06 %, n=7, P=NS for both; ΔEF SMTC vs placebo, n=7, P=NS).

5.3.2.3 Time point: 60 and 120 min after end of infusion

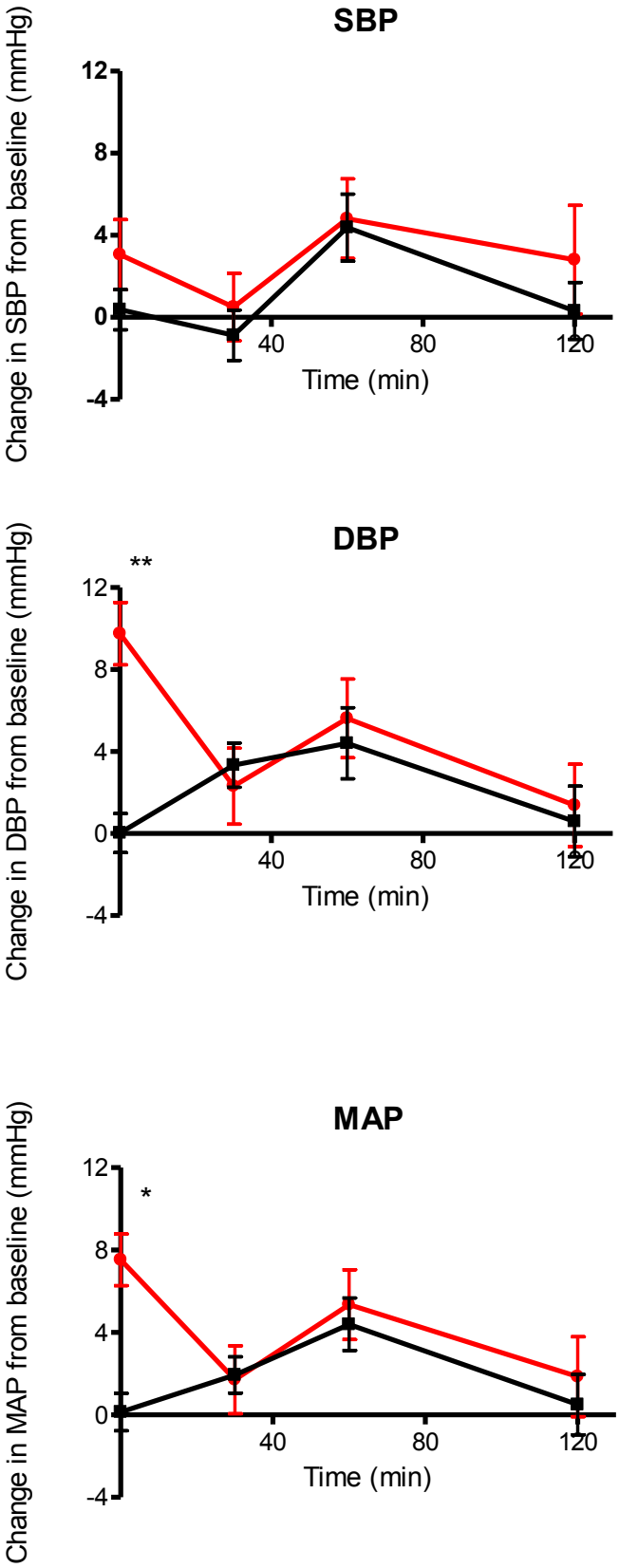
At 60 min after SMTC infusion, when compared to placebo, SMTC no longer had an effect on HR (-3.59±1.20 vs -4.06±1.18 bpm; n=8, P=NS), SBP (4.81±1.94 vs 4.38±1.63 mmHg; n=8, P=NS), DBP (5.63±1.92 vs 4.41±1.73 mmHg; n=8, P=NS) or MAP (5.35±1.69 vs 4.40±1.27 mmHg; n=8, P=NS). At the end of the study, subjects were asked to wait in the patient lounge and were reviewed again at 120min after infusion, to ensure there were no adverse reactions and to repeat measurements of HR and BP. When compared to placebo, SMTC had no effect on HR, SBP, DBP or MAP at this stage either (Figure 5.10).

Figure 5.10: The effect of SMTC 3.0 µmol/Kg 0 to 120 min after the end of infusion for (A) HR (B) BP (C) SV and CO (D) SVR (E) SW. By 30 min SMTC 3.0 µmol/Kg no longer had any effect when compared to placebo. **P<0.001, *P<0.01 (SMTC 3.0 µmol/Kg vs. placebo).

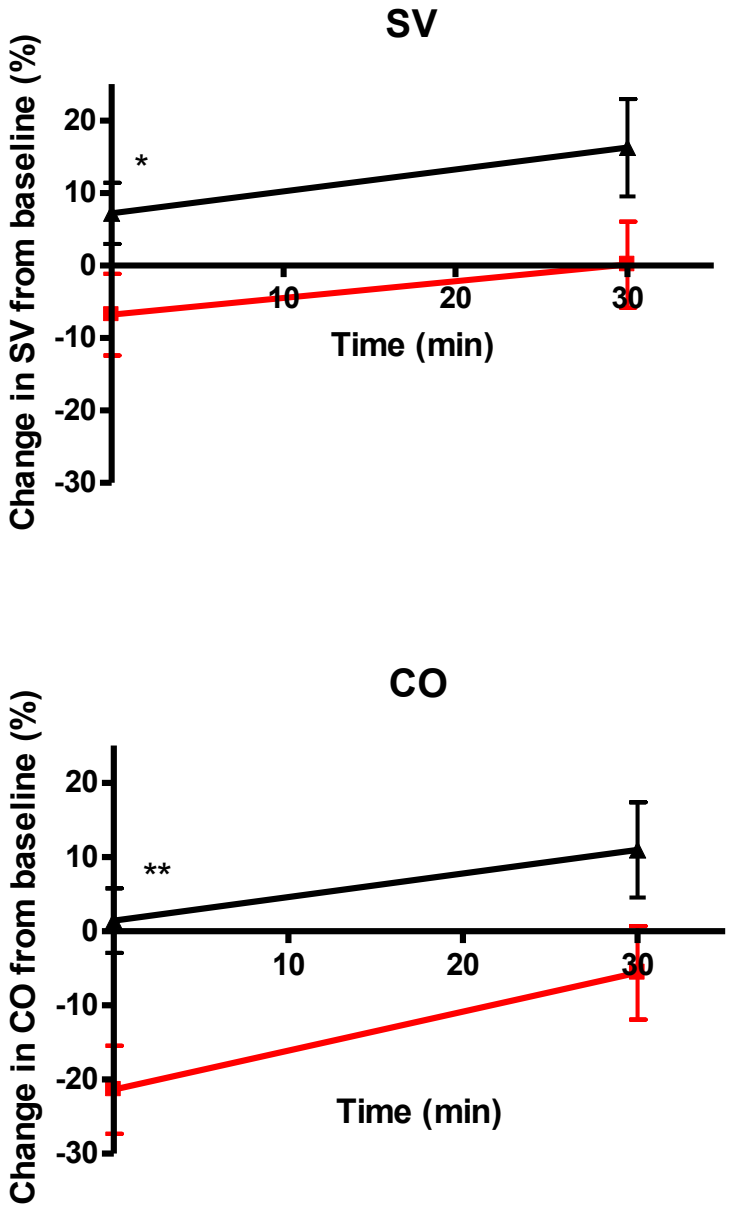
(A)



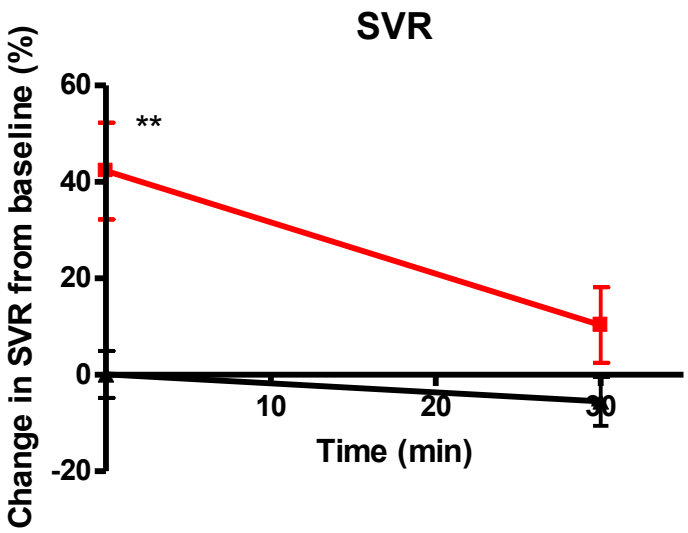
(B)


SBP
Change in SBP from baseline (mmHg)
12
8
4
0
-4
40
80
120
Time (min)
DBP
**
Change in DBP from baseline (mmHg)
12
8
4
0
-4
40
80
120
Time (min)
MAP
*
Change in MAP from baseline (mmHg)
12
8
4
0
-4
40
80
120
Time (min)

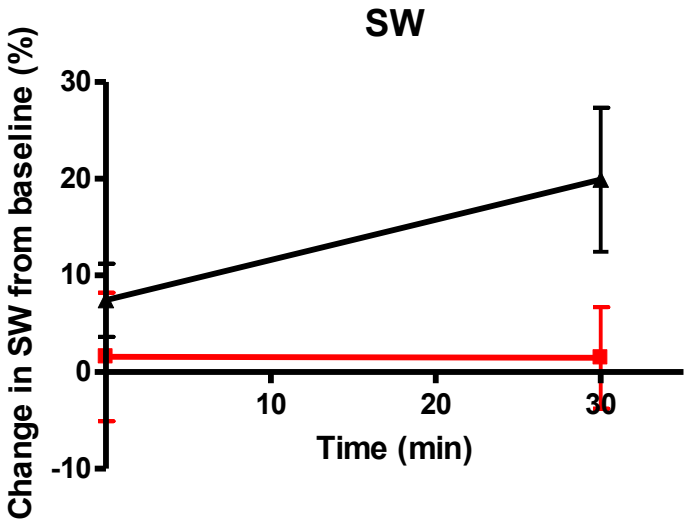
(C)


SV
Change in SV from baseline (%)
20
10
0
-10
-20
-30
*
10
20
30
Time (min)
CO
Change in CO from baseline (%)
20
10
0
-10
-20
-30
**
10
20
30
Time (min)

(D)


SVR
Change in SVR from baseline (%)
60
40
20
0
-20
**
10
20
30
Time (min)

(E)

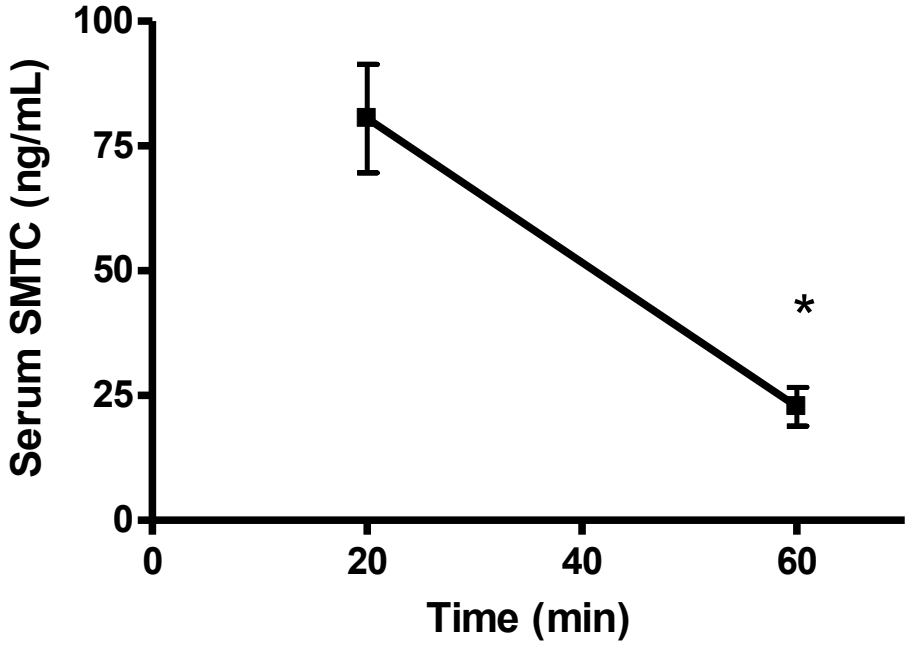

SW
Change in SW from baseline (%)
30
20
10
0
-10
10
20
30
Time (min)

5.3.2.4 Serum SMTC concentration

SMTC was not detected in the blood of any subjects prior to SMTC infusion. SMTC 80.5±10.8 ng/mL was detected in the serum at 20 min after SMTC. This is a SMTC concentration of 0.29 μM. SMTC concentration of approximately 10 μM is optimal for inhibition of nNOS-mediated responses without affecting the eNOS-mediated response to ACh, when infused intra-arterially in animals *in vivo* (Cervenka et al., 2001; Ichihara et al., 1998), therefore these concentrations of SMTC are unlikely to inhibit eNOS.

The concentration decreased to 22.8±3.87 ng/mL at 60 min ($p<0.001$ SMTC concentrations at 20 min vs 60 min) (Figure 5.11). This is consistent with the above results demonstrating the reduced effects of SMTC with time after the end of the infusion.

Figure 5.11: Serum SMTC concentration after the end of infusion. *P<0.001 SMTC concentration at 20 min vs 60 min.



5.4 DISCUSSION

Previously, first-in-human studies from our group with the nNOS-selective inhibitor SMTC showed that the basal regulation of vascular tone in both the forearm and coronary circulations is mediated by nNOS whereas eNOS mediates relaxant responses to pharmacological and shear stress stimuli (Seddon et al., 2009; Seddon et al., 2008). We have now carried out first-in-human studies investigating the effects of systemic nNOS-selective inhibition with SMTC. Systemic SMTC has previously been shown to increase blood pressure in rats *in vivo* (Gozal et al., 1996b; Komers et al., 2000; Wakefield et al., 2003), as has the nNOS-selective inhibitor 7-NI (Ollerstam et al., 1997; Xu et al., 2000).

In our study, SMTC increased DBP and SVR in a dose-dependent manner with the highest dose used of 3 μmol/Kg increasing SVR by 42.2±6.4 %. SMTC also caused a reduction in HR, SV and CO. These results are similar to what has previously been shown with systemic non-selective NOS inhibition with L-NMMA (Brett et al., 1998; Brillante et al., 2009; Hansen et al., 1994b; Haynes et al., 1993; Kiely et al., 1998; Stamler et al., 1994), and imply that the effects that have previously been attributed to eNOS are likely to be largely mediated through nNOS. In support of this is our finding that there was no effect of SMTC on FMD, which suggests that there was no inhibition of eNOS activity at the dose studied. Our maximum dose of SMTC was also up to 8 times smaller than the dose of L-NMMA causing similar haemodynamic effects (Brett et al., 1998), an approximate dose ratio which has been shown to be nNOS-selective in forearm studies (Seddon et al., 2008).

Among the first to use systemic L-NMMA in humans were Haynes and colleagues (Haynes et al., 1993), who found that 3 mg/kg L-NMMA infused systemically over 5 min produced an increase in DBP of 7.1 mmHg and in MAP of 6.9 mmHg, with a decrease in HR of 14 bpm, when compared to placebo, at about 10-15 min after start of infusion. There was no significant increase in SBP in their study. Using a non-invasive bioimpedance method to calculate CO, they found that cardiac index decreased by 25±4 % and SVR increased by 46±12 % (Haynes et al., 1993). Stamler and colleagues used invasive measurement of blood pressure and cardiac output (Fick method) in response to 3 mg/Kg L-NMMA over 3 min. They found a 15.5±1.3 % increase in MAP and a 63.4±8.2 % increase in SVR (Stamler et al., 1994). Both groups supported the notion that through regulation of local vascular tone, NO influences SVR and hence blood pressure.

It is possible that the increase in blood pressure initiates a baroreceptor reflex resulting in withdrawal of sympathetic efferent activity and augmentation of vagal activity, causing a decrease in HR and CO (Bredt et al., 1990; Chowdhary et al., 2000; Schindler et al., 2004; Zanzinger, 1999) . Stamler and colleagues suggested that the effects of L-NMMA on cardiac function cannot be explained solely by the increase in blood pressure as similar changes in blood pressure with phenylephrine (a control vasoconstrictor) did not cause an equivalent decrease in CO (Stamler et al., 1994). Some animal studies have also shown such effects, for example in dogs (Harrison et al., 2000). However it must be noted that phenylephrine may have direct cardiac effects (as a positive inotrope) in addition to being a vasoconstrictor. Hansen and colleagues found that L-NMMA infused systemically increased MAP by 10±2 mmHg and found a reduction in HR and sympathetic nerve activity (measured using

microneurography), but these reflex changes were not different to those evoked by a phenylephrine-induced increase in blood pressure, suggesting that NO is not involved in the attenuation of sympathetic outflow (Hansen et al., 1994a). Charkoudian and colleagues also had similar findings, including no difference in the reflex changes in muscle sympathetic nerve activity with systemic L-NMMA between subjects with high or low baseline sympathetic nerve activity, and concluded that differences in baroreflex control of the peripheral circulation were not major contributors to the different pressor responses between those two groups (Charkoudian et al., 2006). These studies along with some others (Brillante et al., 2009; Cui et al., 2003) go against a major role for NO in central inhibition of sympathetic efferent activity in humans. However others have found a role for NO, with one study showing L-NMMA induced sympathetic activation was masked by an inhibitory effect of arterial baroreflexes indicating that NO is involved in the central regulation of sympathetic outflow in humans (Owlya et al., 1997). Separate from this issue is the question as to whether the hypertensive effect of SMTC might be mediated centrally. Since HR fell (rather than increase) after SMTC, this appears unlikely. It is theoretically also possible that the increase in SVR after SMTC could involve changes in renal renin-angiotensin-aldosterone activity. This requires further study.

The most straight forward explanation for the decrease in SV and CO with SMTC would be that this was due to the increase in SVR (increased afterload). However, it is also possible that there might be a direct cardiac effect of SMTC and that the decrease in CO with systemic nNOS inhibiton may have a multifactorial basis. nNOS-derived NO has a direct effect on the myocardium, it being well established that nNOS is also

located in the cardiac myocytes (Xu et al., 1999) as well as the vessel wall (Kavdia and Popel, 2004) and the peripheral nerves (Toda and Okamura, 2003).

The effect of NO on cardiac contraction has been extensively studied. In the heart, eNOS is mostly found in coronary vascular and endocardial endothelial cells and to a lesser extent in the sarcolemma of cardiac myocytes (Carnicer et al., 2013; Feron et al., 1996). eNOS found in coronary vascular endothelial cells exert paracrine effects on myocardial contraction (Seddon et al., 2007). nNOS is found in the sacroplasmic reticulum of cardiac myocytes, as well as in cardiac autonomic nerves and ganglia (Danson et al., 2005; Xu et al., 1999). The autocrine effects of nNOS appear to include the modulation of basal inotropic state, β-adrenergic responsiveness, and the force-frequency relationship (reviewed in (Seddon et al., 2007). Stimulation of NO from eNOS or nNOS can modulate LV function, particularly diastolic function, and NO has been shown to inhibit myocardial sympathetic (β-adrenergic) responses (Shah and MacCarthy, 2000). NOS inhibition reduces the positive inotropic effect of NO in cardiac muscle (Rassaf et al., 2006) and Prendergast and colleagues found that NO augments the Frank-Starling response by increasing LV compliance in the isolated heart (Prendergast et al., 1997) which occurs for both human and animal models (Paulus and Shah, 1999). It may be that this effect is mediated through nNOS-derived NO as nNOS deficient animals have been shown to have impaired ventricular relaxation (Ashley et al., 2002; Gyurko et al., 2000; Sears et al., 2003). Some of these studies at the same time however found that basal contractility was enhanced, consistent with nNOS-derived NO causing an increased calcium content and greater contraction (Ashley et al., 2002; Sears et al., 2003). Studies on LV myocytes of nNOS deficient mice or with nNOS inhibition showed an enhanced inotropic response to

isoproterenol in LV myocytes, suggesting that the myocardial source of NO involved in the autocrine regulation of β-adrenergic responses may be nNOS. It is possible that the disruption of the nNOS gene is not myocardial-specific, and these mice have been shown to have impaired vagal control of heart rate and an elevated basal sympathetic nerve activity (Choate et al., 2001; Jumrussirikul et al., 1998) which may contribute to the enhanced basal inotropy and the discrepancy between data from *in vivo* mice (Casadei, 2006), which have been shown to produce a smaller inotropic response when compared to wild type mice (Barouch et al., 2002; Dawson et al., 2005).

In the current study, we found no significant effect of SMTC on LV EF or SW. Although more detailed measurements of cardiac function, for example by pressure-volume analysis, would be needed to precisely assess whether systemic SMTC had a direct effect on cardiac contractility, the lack of change in LV SW argues against this possibility. It therefore seems likely that the major driver of the reduction in cardiac output may actually be the increase in SVR.

5.4.1 Heart rate

We found that SMTC reduced heart rate. This may have been secondary to the hypertensive effects of nNOS inhibiton or may even have been a primary effect. Inhibiton of NOS has been shown to have modest bradycardic effects (Pabla and Curtis, 1995) and NO donors elicit a positive chronotropic effect *in vitro* (Musialek et al., 1997) and *in vivo* in animals (Hogan et al., 1999a) and humans (Hogan et al., 1999b). NO is thought to have an important role in the autonomic control of heart rate and this is likely to be nNOS-mediated. As mentioned, nNOS deficient mice have

impaired vagal control of heart rate (Choate et al., 2001; Jumrussirikul et al., 1998). Exercise training induced bradycardia in mice is related to upregulation of nNOS (Danson and Paterson, 2003) and nNOS buffers the increase in heart rate by sympathetic nerve stimulation in rabbits and guinea pigs (Sears et al., 1998). nNOS is also localized in intrinsic cardiac vagal neurons and stellate sympathetic ganglia innervating the sino-atrial node (Herring et al., 2002; Herring and Paterson, 2009). In humans, heart rate increases when systemic L-NMMA is infused with atropine (cholinergic blockade), rather than decreases (L-NMMA alone), and this is abolished by propranolol suggesting this effect is sympathetically mediated (Lepori et al., 2001; Sartori et al., 2005).

5.4.2 Coronary flow

NO also plays a significant role in the regulation of coronary vascular tone. It could also well be possible that the reduction in coronary blood flow seen with NOS inhibition produces a negative inotropic effect, and that this contributes to the reduction in CO. This has been demonstrated in isolated rat hearts (Amrani et al., 1992), as well as in humans *in vivo* (Cotton et al., 2001). However, in our study we did not find any signs of coronary ischaemia clinically or on the subjects' ECG, nor did SMTC effect cardiac left ventricular EF or SW. It is therefore unlikely that changes in coronary flow contributed to the systemic effects that were observed.

5.4.3 Study limitations

Again, our results pertain to healthy men and cannot be extrapolated to women or subjects with cardiovascular risk factors. Due to the limited sample size we cannot exclude a small effect of nNOS to directly reduce cardiac function. This requires further investigation which could be carried out in a cardiac catheterisation suite model with pressure-volume analysis of human left ventricular function.

SMTC has relatively high specificity for nNOS and to be sure that SMTC was acting specifically on nNOS, we measured FMD, an effect mediated through eNOS. However, should more specific inhibitors of nNOS become available for human use it would be advisable to use these to confirm our findings.

5.4.4 Conclusion

In these studies, for the first time in humans we used systemic infusion of SMTC in healthy male volunteers to investigate its effects on systemic haemodynamics *in vivo*. We found that nNOS inhibition produces an increase in SVR and DBP, with a reduction in HR and CO which was previously noted with systemic non-selective NOS inhibition with L-NMMA. This suggests a role for nNOS in the regulation of human SVR and BP.

CHAPTER 6:

GENERAL DISCUSSION

6.1 GENERAL DISCUSSION

In a series of experiments, this thesis aimed to investigate the role of nNOS-derived NO in the regulation of skeletal blood flow during exercise and myocardial blood flow during increased cardiac workload. At a systemic level, the role of nNOS-derived NO on blood pressure and haemodynamics was investigated.

6.1.1 Skeletal blood flow

In the experiments in chapter 3, I used non-selective and selective NOS inhibitors to determine the role of eNOS-derived and nNOS-derived NO in opposing an increase in sympathetically mediated increase in arteriolar tone in the human forearm during handgrip exercise. In further experiments, I investigated the role of eNOS and nNOS-derived NO during reflex sympathetic activation using LBNP +/- handgrip exercise. Experimental data from animals and indirect human studies had suggested that local nNOS-derived NO played an important role in counteracting reflex sympathetic vasoconstriction or contributed to "functional sympatholysis" (the local attenuation of sympathetic vasoconstriction) in the exercising muscle (Sander et al., 2000; Thomas et al., 1998; Thomas and Victor, 1998). We found that despite reducing basal FBF, intra-brachial L-NMMA or SMTC had no significant effect on the increase in FBF or

conduit artery diameter induced by local handgrip exercise. Furthermore, even in the face of increased sympathetic stimulation with LBNP, we found no significant effect of either L-NMMA or SMTC on the FBF response to exercise. Human studies with L-NMMA have not been consistent in demonstrating a role for NO in functional sympatholysis (Dinenno and Joyner, 2003) and our results concur with these findings.

These experiments do not exclude a role for nNOS in exercising muscle. It has been demonstrated that nNOS is abundant in the skeletal muscle (Nakane et al., 1993). Exercise induced reflex sympathetic activation may limit skeletal muscle performance by a direct effect on muscle energetics. α and β adrenergic stimulation could adversely affect muscle efficiency and impair performance by stimulating glycogenolysis and lactate production (Richter et al., 1982). In humans, sympathetic denervation produces important changes in the bioenergetics of the working muscle i.e. it attenuates exercise-induced muscle acidification and causes significant reduction in PCr depletion and ADP concentration. These changes are associated with a significantly reduced pressor response to exercise (Kardos et al., 2000). It may well be that nNOS is involved in this process. Future studies could involve nNOS-selective inhibition with SMTC whilst measuring muscle bioenergetics using magnetic resonance spectroscopy. This technique records signals from high-energy phosphate compounds which are central to energy metabolism *in vivo*. In normal exercising muscle, the hydrolysis of ATP releases the energy necessary for the sliding between myosin and actin proteins, the basis of muscle contraction (Mattei et al., 2004). Different indices are used to illustrate energetics and include the rate of PCr decrease, drop of pH, initial rate of PCr recovery and the recovery of ADP (Chance et al., 1981).

6.1.2 Myocardial blood flow

In the experiments in chapter 4, I investigated the relative contribution of eNOS- and nNOS-derived NO in the regulation of coronary epicardial and microvascular tone in a cardiac catheter laboratory model of increasing metabolic demand as achieved through incremental cardiac pacing. I found that the pacing induced increase in CBF and coronary artery diameter and decrease in CVR was blunted by L-NMMA but not so by SMTC. Pacing likely induces a flow mediated dilatation in the coronary arteries which is regulated by eNOS rather than nNOS, as is the shear stress response in the forearm (Seddon et al., 2009). This effect is consistent with many studies in which L-NMMA attenuated the pacing induced coronary vasodilatation in humans (Quyyumi et al., 1995a).

Our study did not examine the association between nNOS and autonomic mediated changes in metabolic demand in the heart as seen during exercise or mental stress. Seddon and colleagues showed that mental stress-induced vasodilatation in the forearm is significantly attenuated by selective inhibition of nNOS, strongly suggesting a role for local nNOS-derived NO in regulation of microvascular tone during mental stress (Seddon et al., 2008). Whereas the above study was undertaken in the forearm, the effects of mental stress on the coronary circulation may be more relevant from a clinical viewpoint.

In healthy individuals and subjects with angiographically normal coronary arteries, mental stress increases myocardial blood flow through vasodilatation of resistance vessels (Schoder et al., 2000b). This ensures that myocardial blood supply matches the

rise in oxygen demand induced by stress-driven increases in heart rate, blood pressure and myocardial contractility. Mental stress as encountered in daily life or under controlled conditions may contribute to myocardial ischaemia (Krantz et al., 1996). This may account for the increased risk of adverse cardiovascular events (such as myocardial infarction) associated with mental stress (Jiang et al., 1996; Krantz et al., 1999). Coronary artery disease is known to reduce the bioavailability of NO by direct and indirect action on eNOS, however, the influence on nNOS, which may play an important role in regulation of mental stress-induced change in vascular blood flow, remains unclear. Schoder and colleagues demonstrated that in comparison to healthy volunteers, patients with coronary artery disease had a significantly smaller increase in CBF in response to mental stress (Schoder et al., 2000a).

Future studies could examine the role of nNOS in regulation of CBF in response to mental stress in normal coronary arteries and in those with cardiovascular risk factors or established coronary artery disease.

6.1.3 Systemic haemodynamics

In chapter 5, first-in-man experiments were carried out using systemic infusion of SMTC to selectively inhibit nNOS and investigate the effects on systemic haemodynamics. Seddon and colleagues had found, using intra-brachial and intra-coronary infusion of SMTC, that nNOS in involved in the basal regulation of peripheral vascular resistance both in the forearm (Seddon et al., 2008) and coronary (Seddon et al., 2009) circulations. We found that intravenous SMTC increased SVR and blood pressure, whilst SV, CO and heart rate were reduced. Importantly, there was

no effect on FMD, an effect mediated by eNOS. The effects of this dose of SMTC, which we first established in a rising dose response study, were similar to those seen in many previous studies in human's in *viv*o using non-selective NOS inhibition (Haynes et al., 1993; Stamler et al., 1994), which raises the question of whether the effects of NO on basal haemodynamics are attributable to nNOS rather than eNOS.

We raised the question of whether the increase in blood pressure seen with systemic SMTC may not be only due to the effect on the resistance vessels. Indeed, nNOS is also found in the nervous system, where centrally it affects blood pressure, and the kidneys, from which activation of the renin-angiotensin-aldosterone system is important in the regulation of blood pressure. The decrease in cardiac output may well be multifactorial and not only due to a baroreceptor reflex response to increased peripheral vascular resistance, but a primary myocardial effect. NO has both autocrine effects on the heart from the myocyte itself, and paracrine effects from the endothelial cells and nerves. nNOS may play a role via both of these as it is expressed in the myocyte (Xu et al., 1999) and neuronal tissue (Shah and MacCarthy, 2000). However, we found no significant effect of SMTC on LV EF or SW. It therefore seems likely that the major driver of the reduction in cardiac output may actually be the increase in SVR.

6.2 CLINICAL IMPLICATIONS

The results in this thesis may have potential clinical implications. The hallmark of essential hypertension is an increase in peripheral vascular resistance. However, eNOS stimulated responses have been shown to be relatively preserved in essential

hypertension compared to other risk factors for cardiovascular disease, such as diabetes and hypercholesterolaemia (Celermajer et al., 1992; Chowienczyk et al., 1992). Our findings provide a potential explanation for this discrepancy, and raises the important question of whether it is indeed nNOS dysfunction that is the primary abnormality in hypertension. It may be that this is compensated for by increased expression/activity of eNOS, which could explain the relatively preserved eNOS responses seen in hypertension. Such compensatory activity of NOS isoforms is well recognized in eNOS knock-out mice where nNOS may compensate for absence of eNOS (Huang et al., 2002; Meng et al., 1998). Future studies could involve nNOS-selective inhibition with SMTC in patients with hypertension, with the hypothesis that they will reveal a diminished vasoconstrictor response compared to healthy volunteers.

Another clinical scenario where nNOS may be fundamental is heart failure. nNOS is widely found in the cardiac myocytes (Xu et al., 1999) and it has been shown to act as a major modulator of cardiac function and intracellular calcium fluxes (Ashley et al., 2002; Sears et al., 2003). In addition to its effects in the myocyte, it also acts as a modulator of the autonomic control of the cardiovascular system (Herring and Paterson, 2009). Patients with heart failure have a decreased vasomotor response to intra-coronary L-NMMA suggesting that basal release of NO in the coronary circulation is decreased in patients with heart failure (Mohri et al., 1997), an effect which is now believed to be mediated by nNOS (Seddon et al., 2009). Furthermore patients with congestive heart failure had enhanced inotropic response to β-adrenergic agonists after intra-coronary L-NMMA (Hare et al., 1998). Intracoronary L-NMMA also reduced LV contractility in patients with normal LV function, but had no effect in cardiomyopathy patients (Cotton et al., 2001). In animals post-infarction, heart nNOS

expression increases (Bendall et al., 2004; Damy et al., 2003), which was also found to be the case in patients with dilated cardiomyopathy, who furthermore displayed reduced eNOS expression (Damy et al., 2004). It may well be that increased nNOS activity counteracts a decrease in eNOS expression and activity. In this thesis we did not find that any change in the cardiac left ventricular EF or SW with systemic nNOS inhibition. However, future studies could involve more detailed assessment of cardiac function in a cardiac catheter laboratory model by means of pressure-volume analysis. This would investigate the effect that nNOS inhibition had on cardiac contractility, shedding further light on how much is attributable to a primary cardiac effect as opposed to a secondary effect due to the increase in peripheral vascular resistance.

6.3 FUTURE WORK

In this chapter so far, many ideas for future studies have been raised for the different vascular beds being investigated. In this thesis as a whole, forearm and systemic studies were undertaken in healthy men, and coronary studies in patients with angiographically normal coronary arteries (however, this was a combination of men and women, and those with cardiovascular risk factors). It would be interesting to see if there are gender differences in the effect of nNOS inhibition. Potential gender differences in vascular function may be related to the effects of female sex hormones on the vasculature (Orshal and Khalil, 2004), with evidence that sex hormones modify the bioavailability of NO, with total NO production greater in premenopausal women than men (Forte et al., 1998). Future studies could compare the effects of nNOS inhibition on men and women in local vascular beds and on systemic haemodynamics.

Carrying out studies in those with cardiovascular risk factors would also be exciting. As discussed, this would be particularly interesting in subjects with hypertension, in both local vasculature and in systemic nNOS inhibition. Another risk factor which would be fascinating to study be would be patients with diabetes mellitus. nNOS is abundant in the perivascular nerves, and diabetes mellitus is a disease which readily affects the peripheral nervous system. Future studies could investigate the effects of mental stress on vascular tone, an effect which is thought to involve the perivascular nerves, comparing healthy subjects to those with diabetes.

6.4 STUDY LIMITATIONS

Experiments investigating changes in forearm vasomotor tone and systemic haemodynamics were carried out on healthy men only. Further work is required to explore the effect of SMTC on women, and those with cardiovascular risk factors. In contrast, studies investigating coronary vasomotor tone were not performed in healthy volunteers, indeed most of the patients who took part in these studies had at least 1 risk factor for coronary disease, and hence potentially had impaired vasomotor responses (Quyyumi et al., 1995b; Quyyumi et al., 1997; Vita et al., 1990). Further work is required to investigate the effects of nNOS inhibition in the context of these conditions, particularly in hypertension as described above.

SMTC was used in all the studies in this thesis as a selective nNOS inhibitor. In the forearm and coronary studies I used a concentration that was previously shown to reduce basal flow to a similar degree as L-NMMA without inhibiting eNOS mediated responses (Seddon et al., 2009; Seddon et al., 2008). When giving SMTC systemically

I established a dose with similar effects on SVR as previous studies with L-NMMA at an approximate dose ratio which has shown to be nNOS-selective in forearm studies (Seddon et al., 2008), and in the current study did not inhibit FMD. Currently SMTC is the only available nNOS inhibitor available for intra-arterial or intravenous human use, however, others have been used intradermally (Kellogg et al., 2009). Should more specific inhibitors of nNOS become available it would be advisable to use these to confirm our findings.

6.5 CONCLUSION

In this thesis we have found that despite the considerable animal data, and some data in humans, neither NOS isoform plays an obligatory role in functional sympatholysis during exercise in the human forearm *in vivo*. We have also provided the first direct evidence that pacing-induced vasodilatation in the coronary arteries is mediated by eNOS rather than nNOS. And finally, we have carried out first-in-man studies of the effect of nNOS-selective inhibition on systemic haemodynamics. We found an increase in SVR and BP, suggesting a significant role for nNOS in the regulation of these. Furthermore we have established a dose of SMTC which can be given systemically to inhibit nNOS selectively and therefore be used for further studies of nNOS function in humans.

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Publications to date:

1. Shabeeh H, Melikian N, Dworakowski R, Casadei B, Chowienczyk P, Shah AM. Differential role of endothelial vs neuronal nitric oxide synthase in the regulation of coronary blood flow during pacing-induced increases in cardiac workload. *Am J Physiol Heart Circ Physiol*. 2013 May; 304(9):H1277-82.

2. Shabeeh H, Seddon M, Brett S, Melikian N, Casadei B, Shah AM, Chowienczyk P. Sympathetic activation increases NO release from eNOS but neither eNOS nor nNOS play an essential role in exercise hyperaemia in the human forearm. *Am J Physiol Heart Circ Physiol*. 2013 May; 304(9):H1225-30.

3. Seddon M, Melikian N, Dworakowski R, Shabeeh H, Jiang B, Byrne J, Casadei B, Chowienczyk P, Shah A. Effects of neuronal NOS (nNOS) on

human coronary artery diameter and blood flow in vivo. *Circulation.* 2009; May 26:119(20):2656-62.

REFERENCES

Amrani, M., O'Shea, J., Allen, N.J., Harding, S.E., Jayakumar, J., Pepper, J.R., Moncada, S., and Yacoub, M.H. (1992). Role of basal release of nitric oxide on coronary flow and mechanical performance of the isolated rat heart. The Journal of physiology *456*, 681-687.

Anderson, T.J., Uehata, A., Gerhard, M.D., Meredith, I.T., Knab, S., Delagrange, D., Lieberman, E.H., Ganz, P., Creager, M.A., Yeung, A.C.*, et al.* (1995). Close relation of endothelial function in the human coronary and peripheral circulations. Journal of the American College of Cardiology *26*, 1235-1241.

Ashley, E.A., Sears, C.E., Bryant, S.M., Watkins, H.C., and Casadei, B. (2002). Cardiac nitric oxide synthase 1 regulates basal and beta-adrenergic contractility in murine ventricular myocytes. Circulation *105*, 3011-3016.

Babu, B.R., and Griffith, O.W. (1998). N5-(1-Imino-3-butenyl)-L-ornithine. A neuronal isoform selective mechanism-based inactivator of nitric oxide synthase. The Journal of biological chemistry *273*, 8882-8889.

Bachetti, T., Comini, L., Curello, S., Bastianon, D., Palmieri, M., Bresciani, G., Callea, F., and Ferrari, R. (2004). Co-expression and modulation of neuronal and endothelial nitric oxide synthase in human endothelial cells. Journal of molecular and cellular cardiology *37*, 939-945.

Bachmann, S., Bosse, H.M., and Mundel, P. (1995). Topography of nitric oxide synthesis by localizing constitutive NO synthases in mammalian kidney. The American journal of physiology *268*, F885-898.

Barouch, L.A., Harrison, R.W., Skaf, M.W., Rosas, G.O., Cappola, T.P., Kobeissi, Z.A., Hobai, I.A., Lemmon, C.A., Burnett, A.L., O'Rourke, B.*, et al.* (2002). Nitric oxide regulates the heart by spatial confinement of nitric oxide synthase isoforms. Nature *416*, 337-339.

Bauser-Heaton, H.D., and Bohlen, H.G. (2007). Cerebral microvascular dilation during hypotension and decreased oxygen tension: a role for nNOS. American journal of physiology Heart and circulatory physiology *293*, H2193-2201.

Bech, J.N., Nielsen, C.B., and Pedersen, E.B. (1996). Effects of systemic NO synthesis inhibition on RPF, GFR, UNa, and vasoactive hormones in healthy humans. The American journal of physiology *270*, F845-851.

Behrendt, D., and Ganz, P. (2002). Endothelial function. From vascular biology to clinical applications. The American journal of cardiology *90*, 40L-48L.

Bendall, J.K., Damy, T., Ratajczak, P., Loyer, X., Monceau, V., Marty, I., Milliez, P., Robidel, E., Marotte, F., Samuel, J.L.*, et al.* (2004). Role of myocardial neuronal nitric oxide synthase-derived nitric oxide in beta-adrenergic hyporesponsiveness after myocardial infarction-induced heart failure in rat. Circulation *110*, 2368-2375.

Bennett, T., and Gardiner, S. (1996). Nervous Control of Blood Vessels (The Autonomic Nervous System) (Mosby).

Berdeaux, A., Ghaleh, B., Dubois-Rande, J.L., Vigue, B., Drieu La Rochelle, C., Hittinger, L., and Giudicelli, J.F. (1994). Role of vascular endothelium in exercise-induced dilation of large epicardial coronary arteries in conscious dogs. Circulation *89*, 2799-2808.

Bohme, G.A., Bon, C., Lemaire, M., Reibaud, M., Piot, O., Stutzmann, J.M., Doble, A., and Blanchard, J.C. (1993). Altered synaptic plasticity and memory formation in nitric oxide synthase inhibitor-treated rats. Proceedings of the National Academy of Sciences of the United States of America *90*, 9191-9194.

Boulanger, C.M., Heymes, C., Benessiano, J., Geske, R.S., Levy, B.I., and Vanhoutte, P.M. (1998). Neuronal nitric oxide synthase is expressed in rat vascular smooth muscle cells: activation by angiotensin II in hypertension. Circulation research *83*, 1271-1278.

Bredt, D.S., Hwang, P.M., and Snyder, S.H. (1990). Localization of nitric oxide synthase indicating a neural role for nitric oxide. Nature *347*, 768-770.

Brett, S.E., Cockcroft, J.R., Mant, T.G., Ritter, J.M., and Chowienczyk, P.J. (1998). Haemodynamic effects of inhibition of nitric oxide synthase and of L-arginine at rest and during exercise. Journal of hypertension *16*, 429-435.

Brillante, D.G., O'Sullivan, A.J., Johnstone, M.T., and Howes, L.G. (2009). Predictors of inotropic and chronotropic effects of NG-monomethyl-L-arginine. European journal of clinical investigation *39*, 273-279.

Buckwalter, J.B., Taylor, J.C., Hamann, J.J., and Clifford, P.S. (2004). Role of nitric oxide in exercise sympatholysis. J Appl Physiol *97*, 417-423; discussion 416.

Burnett, A.L., Tillman, S.L., Chang, T.S., Epstein, J.I., Lowenstein, C.J., Bredt, D.S., Snyder, S.H., and Walsh, P.C. (1993). Immunohistochemical localization of nitric oxide synthase in the autonomic innervation of the human penis. The Journal of urology *150*, 73-76.

Cardillo, C., Kilcoyne, C.M., Quyyumi, A.A., Cannon, R.O., 3rd, and Panza, J.A. (1997). Role of nitric oxide in the vasodilator response to mental stress in normal subjects. The American journal of cardiology *80*, 1070-1074.

Carnicer, R., Crabtree, M.J., Sivakumaran, V., Casadei, B., and Kass, D.A. (2013). Nitric oxide synthases in heart failure. Antioxidants & redox signaling *18*, 1078-1099.

Casadei, B. (2006). The emerging role of neuronal nitric oxide synthase in the regulation of myocardial function. Experimental physiology *91*, 943-955.

Celermajer, D.S., Sorensen, K.E., Gooch, V.M., Spiegelhalter, D.J., Miller, O.I., Sullivan, I.D., Lloyd, J.K., and Deanfield, J.E. (1992). Non-invasive detection of

endothelial dysfunction in children and adults at risk of atherosclerosis. Lancet *340*, 1111-1115.

Cervenka, L., Kramer, H.J., Maly, J., and Heller, J. (2001). Role of nNOS in regulation of renal function in angiotensin II-induced hypertension. Hypertension *38*, 280-285.

Chance, B., Eleff, S., Leigh, J.S., Jr., Sokolow, D., and Sapega, A. (1981). Mitochondrial regulation of phosphocreatine/inorganic phosphate ratios in exercising human muscle: a gated 31P NMR study. Proceedings of the National Academy of Sciences of the United States of America *78*, 6714-6718.

Charkoudian, N., Joyner, M.J., Barnes, S.A., Johnson, C.P., Eisenach, J.H., Dietz, N.M., and Wallin, B.G. (2006). Relationship between muscle sympathetic nerve activity and systemic hemodynamics during nitric oxide synthase inhibition in humans. American journal of physiology Heart and circulatory physiology *291*, H1378-1383.

Chavoshan, B., Sander, M., Sybert, T.E., Hansen, J., Victor, R.G., and Thomas, G.D. (2002). Nitric oxide-dependent modulation of sympathetic neural control of oxygenation in exercising human skeletal muscle. The Journal of physiology *540*, 377-386.

Chi, O.Z., Liu, X., and Weiss, H.R. (2003). Effects of inhibition of neuronal nitric oxide synthase on NMDA-induced changes in cerebral blood flow and oxygen

consumption. Experimental brain research Experimentelle Hirnforschung Experimentation cerebrale *148*, 256-260.

Choate, J.K., Danson, E.J., Morris, J.F., and Paterson, D.J. (2001). Peripheral vagal control of heart rate is impaired in neuronal NOS knockout mice. American journal of physiology Heart and circulatory physiology *281*, H2310-2317.

Chowdhary, S., Vaile, J.C., Fletcher, J., Ross, H.F., Coote, J.H., and Townend, J.N. (2000). Nitric oxide and cardiac autonomic control in humans. Hypertension *36*, 264-269.

Chowienczyk, P.J., Watts, G.F., Cockcroft, J.R., and Ritter, J.M. (1992). Impaired endothelium-dependent vasodilation of forearm resistance vessels in hypercholesterolaemia. Lancet *340*, 1430-1432.

Clifford, P.S., and Hellsten, Y. (2004). Vasodilatory mechanisms in contracting skeletal muscle. J Appl Physiol *97*, 393-403.

Collier, J.G., and Robinson, B.F. (1974). Comparison of effects of locally infused angiotensin I and II on hand veins and forearm arteries in man: evidence for converting enzyme activity in limb vessels. Clinical science and molecular medicine *47*, 189-192.

Cooper, K.E., Edholm, O.G., and Mottram, R.F. (1955). The blood flow in skin and muscle of the human forearm. The Journal of physiology *128*, 258-267.

Copp, S.W., Hirai, D.M., Schwagerl, P.J., Musch, T.I., and Poole, D.C. (2010). Effects of neuronal nitric oxide synthase inhibition on resting and exercising hindlimb muscle blood flow in the rat. The Journal of physiology *588*, 1321-1331.

Corretti, M.C., Anderson, T.J., Benjamin, E.J., Celermajer, D., Charbonneau, F., Creager, M.A., Deanfield, J., Drexler, H., Gerhard-Herman, M., Herrington, D.*, et al.* (2002). Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. Journal of the American College of Cardiology *39*, 257-265.

Cotton, J.M., Kearney, M.T., MacCarthy, P.A., Grocott-Mason, R.M., McClean, D.R., Heymes, C., Richardson, P.J., and Shah, A.M. (2001). Effects of nitric oxide synthase inhibition on Basal function and the force-frequency relationship in the normal and failing human heart in vivo. Circulation *104*, 2318-2323.

Cottyn, B., Acher, F., Ramassamy, B., Alvey, L., Lepoivre, M., Frapart, Y., Stuehr, D., Mansuy, D., Boucher, J.L., and Vichard, D. (2008). Inhibitory effects of a series of 7-substituted-indazoles toward nitric oxide synthases: particular potency of 1H-indazole-7-carbonitrile. Bioorganic & medicinal chemistry *16*, 5962-5973.

Cox, D.A., Vita, J.A., Treasure, C.B., Fish, R.D., Alexander, R.W., Ganz, P., and Selwyn, A.P. (1989). Atherosclerosis impairs flow-mediated dilation of coronary arteries in humans. Circulation *80*, 458-465.

Cui, J., Zhang, R., Wilson, T.E., Witkowski, S., Crandall, C.G., and Levine, B.D. (2003). Nitric oxide synthase inhibition does not affect regulation of muscle sympathetic nerve activity during head-up tilt. American journal of physiology Heart and circulatory physiology *285*, H2105-2110.

Damkjaer, M., Vafaee, M., Moller, M.L., Braad, P.E., Petersen, H., Hoilund-Carlsen, P.F., and Bie, P. (2010). Renal cortical and medullary blood flow responses to altered NO availability in humans. American journal of physiology Regulatory, integrative and comparative physiology *299*, R1449-1455.

Damy, T., Ratajczak, P., Robidel, E., Bendall, J.K., Oliviero, P., Boczkowski, J., Ebrahimian, T., Marotte, F., Samuel, J.L., and Heymes, C. (2003). Up-regulation of cardiac nitric oxide synthase 1-derived nitric oxide after myocardial infarction in senescent rats. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *17*, 1934-1936.

Danson, E.J., Choate, J.K., and Paterson, D.J. (2005). Cardiac nitric oxide: emerging role for nNOS in regulating physiological function. Pharmacology & therapeutics *106*, 57-74.

Danson, E.J., and Paterson, D.J. (2003). Enhanced neuronal nitric oxide synthase expression is central to cardiac vagal phenotype in exercise-trained mice. The Journal of physiology *546*, 225-232.

Davignon, J., and Ganz, P. (2004). Role of endothelial dysfunction in atherosclerosis. Circulation *109*, III27-32.

Dawson, D., Lygate, C.A., Zhang, M.H., Hulbert, K., Neubauer, S., and Casadei, B. (2005). nNOS gene deletion exacerbates pathological left ventricular remodeling and functional deterioration after myocardial infarction. Circulation *112*, 3729-3737.

Deussen, A., Ohanyan, V., Jannasch, A., Yin, L., and Chilian, W. (2012). Mechanisms of metabolic coronary flow regulation. Journal of molecular and cellular cardiology *52*, 794-801.

Dietz, N.M., Rivera, J.M., Eggener, S.E., Fix, R.T., Warner, D.O., and Joyner, M.J. (1994). Nitric oxide contributes to the rise in forearm blood flow during mental stress in humans. The Journal of physiology *480 (Pt 2)*, 361-368.

Dinenno, F.A., and Joyner, M.J. (2003). Blunted sympathetic vasoconstriction in contracting skeletal muscle of healthy humans: is nitric oxide obligatory? The Journal of physiology *553*, 281-292.

Donald, A.E., Halcox, J.P., Charakida, M., Storry, C., Wallace, S.M., Cole, T.J., Friberg, P., and Deanfield, J.E. (2008). Methodological approaches to optimize reproducibility and power in clinical studies of flow-mediated dilation. Journal of the American College of Cardiology *51*, 1959-1964.

Dorosz, J.L., Lezotte, D.C., Weitzenkamp, D.A., Allen, L.A., and Salcedo, E.E. (2012). Performance of 3-dimensional echocardiography in measuring left ventricular volumes and ejection fraction: a systematic review and meta-analysis. Journal of the American College of Cardiology *59*, 1799-1808.

Doucette, J.W., Corl, P.D., Payne, H.M., Flynn, A.E., Goto, M., Nassi, M., and Segal, J. (1992). Validation of a Doppler guide wire for intravascular measurement of coronary artery flow velocity. Circulation *85*, 1899-1911.

Duffy, S.J., Castle, S.F., Harper, R.W., and Meredith, I.T. (1999). Contribution of vasodilator prostanoids and nitric oxide to resting flow, metabolic vasodilation, and flow-mediated dilation in human coronary circulation. Circulation *100*, 1951-1957.

Duncker, D.J., and Bache, R.J. (2008). Regulation of coronary blood flow during exercise. Physiological reviews *88*, 1009-1086.

Duncker, D.J., Bache, R.J., and Merkus, D. (2012). Regulation of coronary resistance vessel tone in response to exercise. Journal of molecular and cellular cardiology *52*, 802-813.

Egashira, K., Inou, T., Hirooka, Y., Yamada, A., Maruoka, Y., Kai, H., Sugimachi, M., Suzuki, S., and Takeshita, A. (1993). Impaired coronary blood flow response to acetylcholine in patients with coronary risk factors and proximal atherosclerotic lesions. The Journal of clinical investigation *91*, 29-37.

Egashira, K., Katsuda, Y., Mohri, M., Kuga, T., Tagawa, T., Kubota, T., Hirakawa, Y., and Takeshita, A. (1996). Role of endothelium-derived nitric oxide in coronary vasodilatation induced by pacing tachycardia in humans. Circulation research *79*, 331-335.

Endo, T., Imaizumi, T., Tagawa, T., Shiramoto, M., Ando, S., and Takeshita, A. (1994). Role of nitric oxide in exercise-induced vasodilation of the forearm. Circulation *90*, 2886-2890.

Eppel, G.A., Denton, K.M., Malpas, S.C., and Evans, R.G. (2003). Nitric oxide in responses of regional kidney perfusion to renal nerve stimulation and renal ischaemia. Pflugers Archiv : European journal of physiology *447*, 205-213.

Fadel, P.J., Zhao, W., and Thomas, G.D. (2003). Impaired vasomodulation is associated with reduced neuronal nitric oxide synthase in skeletal muscle of ovariectomized rats. The Journal of physiology *549*, 243-253.

Feletou, M., and Vanhoutte, P.M. (1999). The alternative: EDHF. Journal of molecular and cellular cardiology *31*, 15-22.

Feron, O., Belhassen, L., Kobzik, L., Smith, T.W., Kelly, R.A., and Michel, T. (1996). Endothelial nitric oxide synthase targeting to caveolae. Specific interactions with caveolin isoforms in cardiac myocytes and endothelial cells. The Journal of biological chemistry *271*, 22810-22814.

Ferrer, M., Alonso, M.J., Salaices, M., Marin, J., and Balfagon, G. (2000). Increase in neurogenic nitric oxide metabolism by endothelin-1 in mesenteric arteries from hypertensive rats. Journal of cardiovascular pharmacology *36*, 541-547.

Fleming, I., and Busse, R. (1999). Signal transduction of eNOS activation. Cardiovascular research *43*, 532-541.

Forstermann, U., Closs, E.I., Pollock, J.S., Nakane, M., Schwarz, P., Gath, I., and Kleinert, H. (1994). Nitric oxide synthase isozymes. Characterization, purification, molecular cloning, and functions. Hypertension *23*, 1121-1131.

Forte, P., Kneale, B.J., Milne, E., Chowienczyk, P.J., Johnston, A., Benjamin, N., and Ritter, J.M. (1998). Evidence for a difference in nitric oxide biosynthesis between healthy women and men. Hypertension *32*, 730-734.

Furchgott, R.F., and Zawadzki, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature *288*, 373-376.

Furfine, E.S., Harmon, M.F., Paith, J.E., Knowles, R.G., Salter, M., Kiff, R.J., Duffy, C., Hazelwood, R., Oplinger, J.A., and Garvey, E.P. (1994). Potent and selective inhibition of human nitric oxide synthases. Selective inhibition of neuronal nitric oxide synthase by S-methyl-L-thiocitrulline and S-ethyl-L-thiocitrulline. The Journal of biological chemistry *269*, 26677-26683.

Gabbai, F.B. (2001). Effects of nitric oxide synthase blockers on renal function. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association *16 Suppl 1*, 10-13.

Gaglione, A., Hess, O.M., Corin, W.J., Ritter, M., Grimm, J., and Krayenbuehl, H.P. (1987). Is there coronary vasoconstriction after intracoronary beta-adrenergic blockade in patients with coronary artery disease. Journal of the American College of Cardiology *10*, 299-310.

Gamboa, A., Shibao, C., Diedrich, A., Choi, L., Pohar, B., Jordan, J., Paranjape, S., Farley, G., and Biaggioni, I. (2007). Contribution of endothelial nitric oxide to blood pressure in humans. Hypertension *49*, 170-177.

Garland, C.J., Hiley, C.R., and Dora, K.A. (2011). EDHF: spreading the influence of the endothelium. British journal of pharmacology *164*, 839-852.

Ghofrani, H.A., Osterloh, I.H., and Grimminger, F. (2006). Sildenafil: from angina to erectile dysfunction to pulmonary hypertension and beyond. Nature reviews Drug discovery *5*, 689-702.

Gilligan, D.M., Panza, J.A., Kilcoyne, C.M., Waclawiw, M.A., Casino, P.R., and Quyyumi, A.A. (1994). Contribution of endothelium-derived nitric oxide to exercise-induced vasodilation. Circulation *90*, 2853-2858.

Gonzalez, M.A., and Selwyn, A.P. (2003). Endothelial function, inflammation, and prognosis in cardiovascular disease. The American journal of medicine *115 Suppl 8A*, 99S-106S.

Gordon, J.B., Ganz, P., Nabel, E.G., Fish, R.D., Zebede, J., Mudge, G.H., Alexander, R.W., and Selwyn, A.P. (1989). Atherosclerosis influences the vasomotor response of epicardial coronary arteries to exercise. The Journal of clinical investigation *83*, 1946-1952.

Gordon, M.B., Jain, R., Beckman, J.A., and Creager, M.A. (2002). The contribution of nitric oxide to exercise hyperemia in the human forearm. Vasc Med *7*, 163-168.

Gorman, M.W., Tune, J.D., Richmond, K.N., and Feigl, E.O. (2000). Feedforward sympathetic coronary vasodilation in exercising dogs. J Appl Physiol *89*, 1892-1902.

Gozal, D., Torres, J.E., Gozal, Y.M., and Littwin, S.M. (1996a). Effect of nitric oxide synthase inhibition on cardiorespiratory responses in the conscious rat. J Appl Physiol (1985) *81*, 2068-2077.

Gozal, D., Torres, J.E., Gozal, Y.M., and Littwin, S.M. (1996b). Effect of nitric oxide synthase inhibition on cardiorespiratory responses in the conscious rat. Journal of applied physiology *81*, 2068-2077.

Grange, R.W., Isotani, E., Lau, K.S., Kamm, K.E., Huang, P.L., and Stull, J.T. (2001). Nitric oxide contributes to vascular smooth muscle relaxation in contracting fast-twitch muscles. Physiol Genomics *5*, 35-44.

Green, D.J., Bilsborough, W., Naylor, L.H., Reed, C., Wright, J., O'Driscoll, G., and Walsh, J.H. (2005). Comparison of forearm blood flow responses to incremental handgrip and cycle ergometer exercise: relative contribution of nitric oxide. The Journal of physiology *562*, 617-628.

Gyurko, R., Kuhlencordt, P., Fishman, M.C., and Huang, P.L. (2000). Modulation of mouse cardiac function in vivo by eNOS and ANP. American journal of physiology Heart and circulatory physiology *278*, H971-981.

Handy, R.L., Harb, H.L., Wallace, P., Gaffen, Z., Whitehead, K.J., and Moore, P.K. (1996). Inhibition of nitric oxide synthase by 1-(2-trifluoromethylphenyl) imidazole (TRIM) in vitro: antinociceptive and cardiovascular effects. British journal of pharmacology *119*, 423-431.

Hansen, J., Jacobsen, T.N., and Victor, R.G. (1994a). Is nitric oxide involved in the tonic inhibition of central sympathetic outflow in humans? Hypertension *24*, 439-444.

Hansen, J., Jacobsen, T.N., and Victor, R.G. (1994b). Is nitric oxide involved in the tonic inhibition of central sympathetic outflow in humans? Hypertension *24*, 439-444.

Harris, K.F., and Matthews, K.A. (2004). Interactions between autonomic nervous system activity and endothelial function: a model for the development of cardiovascular disease. Psychosomatic medicine *66*, 153-164.

Harrison, R.W., Thakkar, R.N., Senzaki, H., Ekelund, U.E., Cho, E., Kass, D.A., and Hare, J.M. (2000). Relative contribution of preload and afterload to the reduction in cardiac output caused by nitric oxide synthase inhibition with L-N(G)-methylarginine hydrochloride 546C88. Critical care medicine *28*, 1263-1268.

Hasbak, P., Lundby, C., Olsen, N.V., Schifter, S., and Kanstrup, I.L. (2002). Calcitonin gene-related peptide and adrenomedullin release in humans: effects of exercise and hypoxia. Regulatory peptides *108*, 89-95.

Hatanaka, Y., Hobara, N., Honghua, J., Akiyama, S., Nawa, H., Kobayashi, Y., Takayama, F., Gomita, Y., and Kawasaki, H. (2006). Neuronal nitric-oxide synthase inhibition facilitates adrenergic neurotransmission in rat mesenteric resistance arteries. The Journal of pharmacology and experimental therapeutics *316*, 490-497.

Haynes, W.G., Noon, J.P., Walker, B.R., and Webb, D.J. (1993). Inhibition of nitric oxide synthesis increases blood pressure in healthy humans. Journal of hypertension *11*, 1375-1380.

Heiss, H.W., Barmeyer, J., Wink, K., Hell, G., Cerny, F.J., Keul, J., and Reindell, H. (1976). Studies on the regulation of myocardial blood flow in man. I.: Training effects

on blood flow and metabolism of the healthy heart at rest and during standardized heavy exercise. Basic research in cardiology *71*, 658-675.

Herring, N., Danson, E.J., and Paterson, D.J. (2002). Cholinergic control of heart rate by nitric oxide is site specific. News in physiological sciences : an international journal of physiology produced jointly by the International Union of Physiological Sciences and the American Physiological Society *17*, 202-206.

Herring, N., and Paterson, D.J. (2009). Neuromodulators of peripheral cardiac sympatho-vagal balance. Experimental physiology *94*, 46-53.

Hirooka, Y., Shigematsu, H., Kishi, T., Kimura, Y., Ueta, Y., and Takeshita, A. (2003). Reduced nitric oxide synthase in the brainstem contributes to enhanced sympathetic drive in rats with heart failure. Journal of cardiovascular pharmacology *42 Suppl 1*, S111-115.

Hogan, N., Casadei, B., and Paterson, D.J. (1999a). Nitric oxide donors can increase heart rate independent of autonomic activation. J Appl Physiol *87*, 97-103.

Hogan, N., Kardos, A., Paterson, D.J., and Casadei, B. (1999b). Effect of exogenous nitric oxide on baroreflex function in humans. The American journal of physiology *277*, H221-227.

Hokanson, D.E., Sumner, D.S., and Strandness, D.E., Jr. (1975). An electrically calibrated plethysmograph for direct measurement of limb blood flow. IEEE Trans Biomed Eng *22*, 25-29.

Holscher, C., and Rose, S.P. (1992). An inhibitor of nitric oxide synthesis prevents memory formation in the chick. Neuroscience letters *145*, 165-167.

Huang, A., Sun, D., Shesely, E.G., Levee, E.M., Koller, A., and Kaley, G. (2002). Neuronal NOS-dependent dilation to flow in coronary arteries of male eNOS-KO mice. American journal of physiology Heart and circulatory physiology *282*, H429-436.

Huang, Z., Huang, P.L., Panahian, N., Dalkara, T., Fishman, M.C., and Moskowitz, M.A. (1994). Effects of cerebral ischemia in mice deficient in neuronal nitric oxide synthase. Science *265*, 1883-1885.

Ichihara, A., Inscho, E.W., Imig, J.D., and Navar, L.G. (1998). Neuronal nitric oxide synthase modulates rat renal microvascular function. The American journal of physiology *274*, F516-524.

Ignarro, L.J., Buga, G.M., Wood, K.S., Byrns, R.E., and Chaudhuri, G. (1987). Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. ProcNatlAcad Sci USA *84*, 9265-9269.

Jacobs, L.D., Salgo, I.S., Goonewardena, S., Weinert, L., Coon, P., Bardo, D., Gerard, O., Allain, P., Zamorano, J.L., de Isla, L.P.*, et al.* (2006). Rapid online quantification of left ventricular volume from real-time three-dimensional echocardiographic data. European heart journal *27*, 460-468.

Jenkins, C., Bricknell, K., Hanekom, L., and Marwick, T.H. (2004). Reproducibility and accuracy of echocardiographic measurements of left ventricular parameters using real-time three-dimensional echocardiography. Journal of the American College of Cardiology *44*, 878-886.

Jeremy, J.Y., Rowe, D., Emsley, A.M., and Newby, A.C. (1999). Nitric oxide and the proliferation of vascular smooth muscle cells. Cardiovascular research *43*, 580-594.

Jiang, W., Babyak, M., Krantz, D.S., Waugh, R.A., Coleman, R.E., Hanson, M.M., Frid, D.J., McNulty, S., Morris, J.J., O'Connor, C.M.*, et al.* (1996). Mental stress--induced myocardial ischemia and cardiac events. JAMA : the journal of the American Medical Association *275*, 1651-1656.

Joannides, R., Haefeli, W.E., Linder, L., Richard, V., Bakkali, E.H., Thuillez, C., and Luscher, T.F. (1995). Nitric oxide is responsible for flow-dependent dilatation of human peripheral conduit arteries in vivo. Circulation *91*, 1314-1319.

Johnson, J.M., Rowell, L.B., Niederberger, M., and Eisman, M.M. (1974). Human splanchnic and forearm vasoconstrictor responses to reductions of right atrial and aortic pressures. Circulation research *34*, 515-524.

Joyner, M.J., and Dietz, N.M. (2003). Sympathetic vasodilation in human muscle. Acta physiologica Scandinavica *177*, 329-336.

Jumrussirikul, P., Dinerman, J., Dawson, T.M., Dawson, V.L., Ekelund, U., Georgakopoulos, D., Schramm, L.P., Calkins, H., Snyder, S.H., Hare, J.M*., et al.* (1998). Interaction between neuronal nitric oxide synthase and inhibitory G protein activity in heart rate regulation in conscious mice. The Journal of clinical investigation *102*, 1279-1285.

Kardos, A., Taylor, D.J., Thompson, C., Styles, P., Hands, L., Collin, J., and Casadei, B. (2000). Sympathetic denervation of the upper limb improves forearm exercise performance and skeletal muscle bioenergetics. Circulation *101*, 2716-2720.

Kavdia, M., and Popel, A.S. (2004). Contribution of nNOS- and eNOS-derived NO to microvascular smooth muscle NO exposure. J Appl Physiol *97*, 293-301.

Kellogg, D.L., Jr., Liu, Y., Kosiba, I.F., and O'Donnell, D. (1999). Role of nitric oxide in the vascular effects of local warming of the skin in humans. J Appl Physiol *86*, 1185-1190.

Kellogg, D.L., Jr., Zhao, J.L., and Wu, Y. (2008a). Endothelial nitric oxide synthase control mechanisms in the cutaneous vasculature of humans in vivo. American journal of physiology Heart and circulatory physiology *295*, H123-129.

Kellogg, D.L., Jr., Zhao, J.L., and Wu, Y. (2008b). Neuronal nitric oxide synthase control mechanisms in the cutaneous vasculature of humans in vivo. The Journal of physiology *586*, 847-857.

Kellogg, D.L., Jr., Zhao, J.L., and Wu, Y. (2009). Roles of nitric oxide synthase isoforms in cutaneous vasodilation induced by local warming of the skin and whole body heat stress in humans. J Appl Physiol *107*, 1438-1444.

Kharbanda, R.K., and Deanfield, J.E. (2001). Functions of the healthy endothelium. Coronary artery disease *12*, 485-491.

Kiely, D.G., Lee, A.F., Struthers, A.D., and Lipworth, B.J. (1998). Nitric oxide: an important role in the maintenance of systemic and pulmonary vascular tone in man. British journal of clinical pharmacology *46*, 263-266.

Kiss, J.P., and Vizi, E.S. (2001). Nitric oxide: a novel link between synaptic and nonsynaptic transmission. Trends in neurosciences *24*, 211-215.

Kneale, B.J., Chowienczyk, P.J., Brett, S.E., Coltart, D.J., and Ritter, J.M. (2000). Gender differences in sensitivity to adrenergic agonists of forearm resistance vasculature. Journal of the American College of Cardiology *36*, 1233-1238.

Kobayashi, Y.M., Rader, E.P., Crawford, R.W., Iyengar, N.K., Thedens, D.R., Faulkner, J.A., Parikh, S.V., Weiss, R.M., Chamberlain, J.S., Moore, S.A.*, et al.*

(2008). Sarcolemma-localized nNOS is required to maintain activity after mild exercise. Nature *456*, 511-515.

Kobzik, L., Reid, M.B., Bredt, D.S., and Stamler, J.S. (1994). Nitric oxide in skeletal muscle. Nature *372*, 546-548.

Komaru, T., Kanatsuka, H., and Shirato, K. (2000). Coronary microcirculation: physiology and pharmacology. Pharmacology & therapeutics *86*, 217-261.

Komers, R., Lindsley, J.N., Oyama, T.T., Allison, K.M., and Anderson, S. (2000). Role of neuronal nitric oxide synthase (NOS1) in the pathogenesis of renal hemodynamic changes in diabetes. American journal of physiology Renal physiology *279*, F573-583.

Krantz, D.S., Kop, W.J., Santiago, H.T., and Gottdiener, J.S. (1996). Mental stress as a trigger of myocardial ischemia and infarction. Cardiology clinics *14*, 271-287.

Krantz, D.S., Santiago, H.T., Kop, W.J., Bairey Merz, C.N., Rozanski, A., and Gottdiener, J.S. (1999). Prognostic value of mental stress testing in coronary artery disease. The American journal of cardiology *84*, 1292-1297.

Kuo, L., Chilian, W.M., and Davis, M.J. (1991). Interaction of pressure- and flow-induced responses in porcine coronary resistance vessels. The American journal of physiology *261*, H1706-1715.

Lahera, V., Salom, M.G., Miranda-Guardiola, F., Moncada, S., and Romero, J.C. (1991). Effects of NG-nitro-L-arginine methyl ester on renal function and blood pressure. The American journal of physiology *261*, F1033-1037.

Lai, Y., Thomas, G.D., Yue, Y., Yang, H.T., Li, D., Long, C., Judge, L., Bostick, B., Chamberlain, J.S., Terjung, R.L.*, et al.* (2009). Dystrophins carrying spectrin-like repeats 16 and 17 anchor nNOS to the sarcolemma and enhance exercise performance in a mouse model of muscular dystrophy. The Journal of clinical investigation *119*, 624-635.

Landmesser, U., Hornig, B., and Drexler, H. (2004). Endothelial function: a critical determinant in atherosclerosis? Circulation *109*, II27-33.

Lefroy, D.C., Crake, T., Uren, N.G., Davies, G.J., and Maseri, A. (1993). Effect of inhibition of nitric oxide synthesis on epicardial coronary artery caliber and coronary blood flow in humans. Circulation *88*, 43-54.

Lepori, M., Sartori, C., Duplain, H., Nicod, P., and Scherrer, U. (2001). Interaction between cholinergic and nitrergic vasodilation: a novel mechanism of blood pressure control. Cardiovascular research *51*, 767-772.

Li, H., and Forstermann, U. (2000). Nitric oxide in the pathogenesis of vascular disease. J Pathol *190*, 244-254.

Liu, Y., Bubolz, A.H., Mendoza, S., Zhang, D.X., and Gutterman, D.D. (2011). H2O2 is the transferrable factor mediating flow-induced dilation in human coronary arterioles. Circulation research *108*, 566-573.

Longhurst, J., Capone, R.J., Mason, D.T., and Zelis, R. (1974). Comparison of blood flow measured by plethysmograph and flowmeter during steady state forearm exercise. Circulation *49*, 535-540.

Ludmer, P.L., Selwyn, A.P., Shook, T.L., Wayne, R.R., Mudge, G.H., Alexander, R.W., and Ganz, P. (1986). Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. The New England journal of medicine *315*, 1046-1051.

Lundberg, J.M. (1996). Pharmacology of cotransmission in the autonomic nervous system: integrative aspects on amines, neuropeptides, adenosine triphosphate, amino acids and nitric oxide. Pharmacological reviews *48*, 113-178.

Macho, P., Hintze, T.H., and Vatner, S.F. (1981). Regulation of large coronary arteries by increases in myocardial metabolic demands in conscious dogs. Circulation research *49*, 594-599.

Majid, D.S., and Navar, L.G. (2001). Nitric oxide in the control of renal hemodynamics and excretory function. American journal of hypertension *14*, 74S-82S.

Majid, D.S., Williams, A., and Navar, L.G. (1993). Inhibition of nitric oxide synthesis attenuates pressure-induced natriuretic responses in anesthetized dogs. The American journal of physiology *264*, F79-87.

Mattei, J.P., Bendahan, D., and Cozzone, P. (2004). P-31 magnetic resonance spectroscopy. A tool for diagnostic purposes and pathophysiological insights in muscle diseases. Reumatismo *56*, 9-14.

Mattson, D.L., Roman, R.J., and Cowley, A.W., Jr. (1992). Role of nitric oxide in renal papillary blood flow and sodium excretion. Hypertension *19*, 766-769.

Meng, W., Ayata, C., Waeber, C., Huang, P.L., and Moskowitz, M.A. (1998). Neuronal NOS-cGMP-dependent ACh-induced relaxation in pial arterioles of endothelial NOS knockout mice. The American journal of physiology *274*, H411-415.

Merkus, D., Houweling, B., Zarbanoui, A., and Duncker, D.J. (2004). Interaction between prostanoids and nitric oxide in regulation of systemic, pulmonary, and coronary vascular tone in exercising swine. American journal of physiology Heart and circulatory physiology *286*, H1114-1123.

Michel, T., and Feron, O. (1997). Nitric oxide synthases: which, where, how, and why? The Journal of clinical investigation *100*, 2146-2152.

Miyauchi, T., and Masaki, T. (1999). Pathophysiology of endothelin in the cardiovascular system. Annual review of physiology *61*, 391-415.

Mohri, M., Egashira, K., Tagawa, T., Kuga, T., Tagawa, H., Harasawa, Y., Shimokawa, H., and Takeshita, A. (1997). Basal release of nitric oxide is decreased in the coronary circulation in patients with heart failure. Hypertension *30*, 50-56.

Mombouli, J.V., and Vanhoutte, P.M. (1999). Endothelial dysfunction: from physiology to therapy. Journal of molecular and cellular cardiology *31*, 61-74.

Moncada, S., and Higgs, A. (1993). The L-arginine-nitric oxide pathway. The New England journal of medicine *329*, 2002-2012.

Moncada, S., and Higgs, E.A. (2006). Nitric oxide and the vascular endothelium. Handbook of experimental pharmacology, 213-254.

Mount, P.F., and Power, D.A. (2006). Nitric oxide in the kidney: functions and regulation of synthesis. Acta Physiol (Oxf) *187*, 433-446.

Musialek, P., Lei, M., Brown, H.F., Paterson, D.J., and Casadei, B. (1997). Nitric oxide can increase heart rate by stimulating the hyperpolarization-activated inward current, I(f). Circulation research *81*, 60-68.

Nabel, E.G., Selwyn, A.P., and Ganz, P. (1990a). Large coronary arteries in humans are responsive to changing blood flow: an endothelium-dependent mechanism that fails in patients with atherosclerosis. Journal of the American College of Cardiology *16*, 349-356.

Nabel, E.G., Selwyn, A.P., and Ganz, P. (1990b). Paradoxical narrowing of atherosclerotic coronary arteries induced by increases in heart rate. Circulation *81*, 850-859.

Nakane, M., Schmidt, H.H., Pollock, J.S., Forstermann, U., and Murad, F. (1993). Cloned human brain nitric oxide synthase is highly expressed in skeletal muscle. FEBS letters *316*, 175-180.

Narayanan, K., Spack, L., McMillan, K., Kilbourn, R.G., Hayward, M.A., Masters, B.S., and Griffith, O.W. (1995). S-alkyl-L-thiocitrullines. Potent stereoselective inhibitors of nitric oxide synthase with strong pressor activity in vivo. The Journal of biological chemistry *270*, 11103-11110.

Nelson, R.J., Demas, G.E., Huang, P.L., Fishman, M.C., Dawson, V.L., Dawson, T.M., and Snyder, S.H. (1995). Behavioural abnormalities in male mice lacking neuronal nitric oxide synthase. Nature *378*, 383-386.

O'Brien, E., Asmar, R., Beilin, L., Imai, Y., Mallion, J.M., Mancia, G., Mengden, T., Myers, M., Padfield, P., Palatini, P.*, et al.* (2003). European Society of Hypertension recommendations for conventional, ambulatory and home blood pressure measurement. Journal of hypertension *21*, 821-848.

O'Dell, T.J., Hawkins, R.D., Kandel, E.R., and Arancio, O. (1991). Tests of the roles of two diffusible substances in long-term potentiation: evidence for nitric oxide as a

possible early retrograde messenger. Proceedings of the National Academy of Sciences of the United States of America *88*, 11285-11289.

Okamura, T., Yoshida, K., and Toda, N. (1995). Nitroxidergic innervation in dog and monkey renal arteries. Hypertension *25*, 1090-1095.

Oliver, J.J., Melville, V.P., and Webb, D.J. (2006). Effect of regular phosphodiesterase type 5 inhibition in hypertension. Hypertension *48*, 622-627.

Ollerstam, A., Pittner, J., Persson, A.E., and Thorup, C. (1997). Increased blood pressure in rats after long-term inhibition of the neuronal isoform of nitric oxide synthase. The Journal of clinical investigation *99*, 2212-2218.

Orshal, J.M., and Khalil, R.A. (2004). Gender, sex hormones, and vascular tone. American journal of physiology Regulatory, integrative and comparative physiology *286*, R233-249.

Ortiz, P.A., and Garvin, J.L. (2002). Role of nitric oxide in the regulation of nephron transport. American journal of physiology Renal physiology *282*, F777-784.

Owlya, R., Vollenweider, L., Trueb, L., Sartori, C., Lepori, M., Nicod, P., and Scherrer, U. (1997). Cardiovascular and sympathetic effects of nitric oxide inhibition at rest and during static exercise in humans. Circulation *96*, 3897-3903.

Pabla, R., and Curtis, M.J. (1995). Effects of NO modulation on cardiac arrhythmias in the rat isolated heart. Circulation research *77*, 984-992.

Pallone, T.L., and Mattson, D.L. (2002). Role of nitric oxide in regulation of the renal medulla in normal and hypertensive kidneys. Current opinion in nephrology and hypertension *11*, 93-98.

Palmer, R.M., Ferrige, A.G., and Moncada, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature *327*, 524-526.

Paulus, W.J., and Shah, A.M. (1999). NO and cardiac diastolic function. Cardiovascular research *43*, 595-606.

Percival, J.M., Anderson, K.N., Gregorevic, P., Chamberlain, J.S., and Froehner, S.C. (2008). Functional deficits in nNOSmu-deficient skeletal muscle: myopathy in nNOS knockout mice. PloS one *3*, e3387.

Percival, J.M., Anderson, K.N., Huang, P., Adams, M.E., and Froehner, S.C. (2010). Golgi and sarcolemmal neuronal NOS differentially regulate contraction-induced fatigue and vasoconstriction in exercising mouse skeletal muscle. The Journal of clinical investigation *120*, 816-826.

Plochocka-Zulinska, D., and Krukoff, T.L. (1997). Increased gene expression of neuronal nitric oxide synthase in brain of adult spontaneously hypertensive rats. Brain research Molecular brain research *48*, 291-297.

Prendergast, B.D., Sagach, V.F., and Shah, A.M. (1997). Basal release of nitric oxide augments the Frank-Starling response in the isolated heart. Circulation *96*, 1320-1329.

Qadri, F., Arens, T., Schwarz, E.C., Hauser, W., Dendorfer, A., and Dominiak, P. (2003). Brain nitric oxide synthase activity in spontaneously hypertensive rats during the development of hypertension. Journal of hypertension *21*, 1687-1694.

Quyyumi, A.A., Dakak, N., Andrews, N.P., Gilligan, D.M., Panza, J.A., and Cannon, R.O., 3rd (1995a). Contribution of nitric oxide to metabolic coronary vasodilation in the human heart. Circulation *92*, 320-326.

Quyyumi, A.A., Dakak, N., Andrews, N.P., Husain, S., Arora, S., Gilligan, D.M., Panza, J.A., and Cannon, R.O., 3rd (1995b). Nitric oxide activity in the human coronary circulation. Impact of risk factors for coronary atherosclerosis. The Journal of clinical investigation *95*, 1747-1755.

Quyyumi, A.A., Mulcahy, D., Andrews, N.P., Husain, S., Panza, J.A., and Cannon, R.O., 3rd (1997). Coronary vascular nitric oxide activity in hypertension and hypercholesterolemia. Comparison of acetylcholine and substance P. Circulation *95*, 104-110.

Rand, M.J. (1992). Nitrergic transmission: nitric oxide as a mediator of non-adrenergic, non-cholinergic neuro-effector transmission. Clinical and experimental pharmacology & physiology *19*, 147-169.

Rassaf, T., Poll, L.W., Brouzos, P., Lauer, T., Totzeck, M., Kleinbongard, P., Gharini, P., Andersen, K., Schulz, R., Heusch, G.*, et al.* (2006). Positive effects of nitric oxide on left ventricular function in humans. European heart journal *27*, 1699-1705.

Rees, D.D., Palmer, R.M., and Moncada, S. (1989). Role of endothelium-derived nitric oxide in the regulation of blood pressure. Proceedings of the National Academy of Sciences of the United States of America *86*, 3375-3378.

Remensnyder, J.P., Mitchell, J.H., and Sarnoff, S.J. (1962). Functional sympatholysis during muscular activity. Observations on influence of carotid sinus on oxygen uptake. Circulation research *11*, 370-380.

Richter, E.A., Ruderman, N.B., and Galbo, H. (1982). Alpha and beta adrenergic effects on metabolism in contracting, perfused muscle. Acta physiologica Scandinavica *116*, 215-222.

Sander, M., Chavoshan, B., Harris, S.A., Iannaccone, S.T., Stull, J.T., Thomas, G.D., and Victor, R.G. (2000). Functional muscle ischemia in neuronal nitric oxide synthase-deficient skeletal muscle of children with Duchenne muscular dystrophy. Proceedings of the National Academy of Sciences of the United States of America *97*, 13818-13823.

Sartori, C., Lepori, M., and Scherrer, U. (2005). Interaction between nitric oxide and the cholinergic and sympathetic nervous system in cardiovascular control in humans. Pharmacology & therapeutics *106*, 209-220.

Schindler, C., Dobrev, D., Grossmann, M., Francke, K., Pittrow, D., and Kirch, W. (2004). Mechanisms of beta-adrenergic receptor-mediated venodilation in humans. Clinical pharmacology and therapeutics *75*, 49-59.

Schoder, H., Silverman, D.H., Campisi, R., Karpman, H., Phelps, M.E., Schelbert, H.R., and Czernin, J. (2000a). Effect of mental stress on myocardial blood flow and vasomotion in patients with coronary artery disease. Journal of nuclear medicine : official publication, Society of Nuclear Medicine *41*, 11-16.

Schoder, H., Silverman, D.H., Campisi, R., Sayre, J.W., Phelps, M.E., Schelbert, H.R., and Czernin, J. (2000b). Regulation of myocardial blood flow response to mental stress in healthy individuals. American journal of physiology Heart and circulatory physiology *278*, H360-366.

Schuman, E.M., and Madison, D.V. (1994). Nitric oxide and synaptic function. Annual review of neuroscience *17*, 153-183.

Sears, C.E., Bryant, S.M., Ashley, E.A., Lygate, C.A., Rakovic, S., Wallis, H.L., Neubauer, S., Terrar, D.A., and Casadei, B. (2003). Cardiac neuronal nitric oxide

synthase isoform regulates myocardial contraction and calcium handling. Circulation research *92*, e52-59.

Sears, C.E., Choate, J.K., and Paterson, D.J. (1998). Effect of nitric oxide synthase inhibition on the sympatho-vagal contol of heart rate. Journal of the autonomic nervous system *73*, 63-73.

Seddon, M., Melikian, N., Dworakowski, R., Shabeeh, H., Jiang, B., Byrne, J., Casadei, B., Chowienczyk, P., and Shah, A.M. (2009). Effects of neuronal nitric oxide synthase on human coronary artery diameter and blood flow in vivo. Circulation *119*, 2656-2662.

Seddon, M., Shah, A.M., and Casadei, B. (2007). Cardiomyocytes as effectors of nitric oxide signalling. Cardiovascular research *75*, 315-326.

Seddon, M.D., Chowienczyk, P.J., Brett, S.E., Casadei, B., and Shah, A.M. (2008). Neuronal nitric oxide synthase regulates basal microvascular tone in humans in vivo. Circulation *117*, 1991-1996.

Shah, A.M., and MacCarthy, P.A. (2000). Paracrine and autocrine effects of nitric oxide on myocardial function. Pharmacology & therapeutics *86*, 49-86.

Shah, A.M., Vallance, P., and Harrison, D. (1999). NO in the cardiovascular system. Cardiovascular research *43*, 507-508.

Solomon, H., Man, J.W., and Jackson, G. (2003). Erectile dysfunction and the cardiovascular patient: endothelial dysfunction is the common denominator. Heart *89*, 251-253.

Sorensen, K.E., Celermajer, D.S., Spiegelhalter, D.J., Georgakopoulos, D., Robinson, J., Thomas, O., and Deanfield, J.E. (1995). Non-invasive measurement of human endothelium dependent arterial responses: accuracy and reproducibility. Br Heart J *74*, 247-253.

Stamler, J.S., Loh, E., Roddy, M.A., Currie, K.E., and Creager, M.A. (1994). Nitric oxide regulates basal systemic and pulmonary vascular resistance in healthy humans. Circulation *89*, 2035-2040.

Straub, V., and Campbell, K.P. (1997). Muscular dystrophies and the dystrophin-glycoprotein complex. Current opinion in neurology *10*, 168-175.

Sugeng, L., Mor-Avi, V., Weinert, L., Niel, J., Ebner, C., Steringer-Mascherbauer, R., Schmidt, F., Galuschky, C., Schummers, G., Lang, R.M.*, et al.* (2006). Quantitative assessment of left ventricular size and function: side-by-side comparison of real-time three-dimensional echocardiography and computed tomography with magnetic resonance reference. Circulation *114*, 654-661.

Sundlof, G., and Wallin, B.G. (1978). Effect of lower body negative pressure on human muscle nerve sympathetic activity. The Journal of physiology *278*, 525-532.

Taddei, S., Virdis, A., Ghiadoni, L., Sudano, I., Notari, M., and Salvetti, A. (1999). Vasoconstriction to endogenous endothelin-1 is increased in the peripheral circulation of patients with essential hypertension. Circulation *100*, 1680-1683.

Thomas, G.D., Sander, M., Lau, K.S., Huang, P.L., Stull, J.T., and Victor, R.G. (1998). Impaired metabolic modulation of alpha-adrenergic vasoconstriction in dystrophin-deficient skeletal muscle. Proceedings of the National Academy of Sciences of the United States of America *95*, 15090-15095.

Thomas, G.D., and Victor, R.G. (1998). Nitric oxide mediates contraction-induced attenuation of sympathetic vasoconstriction in rat skeletal muscle. The Journal of physiology *506 (Pt 3)*, 817-826.

Toda, N., Ayajiki, K., and Okamura, T. (2009). Control of systemic and pulmonary blood pressure by nitric oxide formed through neuronal nitric oxide synthase. Journal of hypertension *27*, 1929-1940.

Toda, N., and Okamura, T. (1989). Endothelium-dependent and -independent responses to vasoactive substances of isolated human coronary arteries. The American journal of physiology *257*, H988-995.

Toda, N., and Okamura, T. (1990). Modification by L-NG-monomethyl arginine (L-NMMA) of the response to nerve stimulation in isolated dog mesenteric and cerebral arteries. Japanese journal of pharmacology *52*, 170-173.

Toda, N., and Okamura, T. (1996). Nitroxidergic nerve: regulation of vascular tone and blood flow in the brain. Journal of hypertension *14*, 423-434.

Toda, N., and Okamura, T. (2003). The pharmacology of nitric oxide in the peripheral nervous system of blood vessels. Pharmacological reviews *55*, 271-324.

Toda, N., and Okamura, T. (2011). Modulation of renal blood flow and vascular tone by neuronal nitric oxide synthase-derived nitric oxide. Journal of vascular research *48*, 1-10.

Tost, H., Hably, C., Lengyel, M., Gogl, A., Lendvai, A., and Bartha, J. (2000). Effect of nitric oxide synthase inhibition on renal circulation and excretory function in anaesthetized rats. Experimental physiology *85*, 791-800.

Tousoulis, D., Tentolouris, C., Crake, T., Toutouzas, P., and Davies, G. (1997). Basal and flow-mediated nitric oxide production by atheromatous coronary arteries. Journal of the American College of Cardiology *29*, 1256-1262.

Tschakovsky, M.E., Sujirattanawimol, K., Ruble, S.B., Valic, Z., and Joyner, M.J. (2002). Is sympathetic neural vasoconstriction blunted in the vascular bed of exercising human muscle? The Journal of physiology *541*, 623-635.

Tsui, J.C., Souza, D.S., Filbey, D., Karlsson, M.G., and Dashwood, M.R. (2002). Localization of nitric oxide synthase in saphenous vein grafts harvested with a novel

"no-touch" technique: potential role of nitric oxide contribution to improved early graft patency rates. J Vasc Surg *35*, 356-362.

Vallance, P., and Chan, N. (2001). Endothelial function and nitric oxide: clinical relevance. Heart *85*, 342-350.

Vallance, P., Collier, J., and Moncada, S. (1989). Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man. Lancet *2*, 997-1000.

Vallon, V., Traynor, T., Barajas, L., Huang, Y.G., Briggs, J.P., and Schnermann, J. (2001). Feedback control of glomerular vascular tone in neuronal nitric oxide synthase knockout mice. Journal of the American Society of Nephrology : JASN *12*, 1599-1606.

Verma, S., and Anderson, T.J. (2002). Fundamentals of endothelial function for the clinical cardiologist. Circulation *105*, 546-549.

Vita, J.A., Treasure, C.B., Nabel, E.G., McLenachan, J.M., Fish, R.D., Yeung, A.C., Vekshtein, V.I., Selwyn, A.P., and Ganz, P. (1990). Coronary vasomotor response to acetylcholine relates to risk factors for coronary artery disease. Circulation *81*, 491-497.

Wakefield, I.D., March, J.E., Kemp, P.A., Valentin, J.P., Bennett, T., and Gardiner, S.M. (2003). Comparative regional haemodynamic effects of the nitric oxide synthase

inhibitors, S-methyl-L-thiocitrulline and L-NAME, in conscious rats. British journal of pharmacology *139*, 1235-1243.

Walker, H.A., Jackson, G., Ritter, J.M., and Chowienczyk, P.J. (2001). Assessment of forearm vasodilator responses to acetylcholine and albuterol by strain gauge plethysmography: reproducibility and influence of strain gauge placement. British journal of clinical pharmacology *51*, 225-229.

Wang, Y., Liu, X.F., Cornish, K.G., Zucker, I.H., and Patel, K.P. (2005). Effects of nNOS antisense in the paraventricular nucleus on blood pressure and heart rate in rats with heart failure. American journal of physiology Heart and circulatory physiology *288*, H205-213.

Webb, G.D., Lim, L.H., Oh, V.M., El Oakley, R., Lee, C.N., Wong, P.S., Aye, W.M., Chan, E.S., and Moore, P.K. (2006). Expression of neuronal nitric oxide synthase in the internal thoracic artery and saphenous vein. The Journal of thoracic and cardiovascular surgery *132*, 1131-1136.

Whitney, R.J. (1953). The measurement of volume changes in human limbs. The Journal of physiology *121*, 1-27.

Wilcox, C.S., and Welch, W.J. (1998). Macula densa nitric oxide synthase: expression, regulation, and function. Kidney international Supplement *67*, S53-57.

Wilkins, R., and Bradley, S. (1946). Changes in arterial and venous blood pressure and flow distal to a cuff inflated on the human arm. The American journal of physiology *147*, 260-269.

Wilson, J.R., and Kapoor, S. (1993). Contribution of endothelium-derived relaxing factor to exercise-induced vasodilation in humans. J Appl Physiol *75*, 2740-2744.

Wray, D.W., Witman, M.A., Ives, S.J., McDaniel, J., Fjeldstad, A.S., Trinity, J.D., Conklin, J.D., Supiano, M.A., and Richardson, R.S. (2011). Progressive handgrip exercise: evidence of nitric oxide-dependent vasodilation and blood flow regulation in humans. American journal of physiology Heart and circulatory physiology *300*, H1101-1107.

Xu, K.Y., Huso, D.L., Dawson, T.M., Bredt, D.S., and Becker, L.C. (1999). Nitric oxide synthase in cardiac sarcoplasmic reticulum. Proceedings of the National Academy of Sciences of the United States of America *96*, 657-662.

Xu, L., Carter, E.P., Ohara, M., Martin, P.Y., Rogachev, B., Morris, K., Cadnapaphornchai, M., Knotek, M., and Schrier, R.W. (2000). Neuronal nitric oxide synthase and systemic vasodilation in rats with cirrhosis. American journal of physiology Renal physiology *279*, F1110-1115.

Yeung, A.C., Vekshtein, V.I., Krantz, D.S., Vita, J.A., Ryan, T.J., Jr., Ganz, P., and Selwyn, A.P. (1991). The effect of atherosclerosis on the vasomotor response of

coronary arteries to mental stress. The New England journal of medicine *325*, 1551-1556.

Zanzinger, J. (1999). Role of nitric oxide in the neural control of cardiovascular function. Cardiovascular research *43*, 639-649.

Zeiher, A.M., Drexler, H., Wollschlager, H., and Just, H. (1991). Modulation of coronary vasomotor tone in humans. Progressive endothelial dysfunction with different early stages of coronary atherosclerosis. Circulation *83*, 391-401.

Zhou, L., and Zhu, D.Y. (2009). Neuronal nitric oxide synthase: structure, subcellular localization, regulation, and clinical implications. Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society *20*, 223-230.

Zoccali, C. (2007). The endothelium as a target in renal diseases. Journal of nephrology *20 Suppl 12*, S39-44.

Zoller, R.P., Mark, A.L., Abboud, F.M., Schmid, P.G., and Heistad, D.D. (1972). The role of low pressure baroreceptors in reflex vasoconstrictor responses in man. The Journal of clinical investigation *51*, 2967-2972.